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Canada

OAT β-GLUCAN:

BIOCHEMISTRY, STRUCTURE AND GENETIC VARIATION

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Thesis submitted to the Department of Biochemistry
in partial fulfillment of the requirements of the
degree of Doctor of Philosophy

University of Ottawa
Ottawa, Ontario, Canada

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ABSTRACT

An enzymatic assay designed for measurement of ß-glucan in barley was modified to allow measurement of total ß-glucan content in oats by manipulating the grinding and incubation protocol. Using the modified enzymatic assay, the range of genetic and environmental variation of ß-glucan content in Canadian domestic and breeder's lines of oats was assessed using several cultivars grown in 5 locations in eastern Canada in 3 growing seasons. Analysis of variance indicated that the predominant source of variation was genetic. A second assay using Flow Injection Analysis (FIA) to measure ß-glucan was also evaluated. Although a high correlation was observed for the results of the two methods (r=0.90), the results obtained using FIA tended to be somewhat lower than those obtained using the modified enzymatic assay: the enzymatic assay was judged to be more accurate for estimation of total ß-glucan in oats. Nevertheless, because of its greater speed and simplicity, FIA would be a valuable screening tool for routine applications. Using the enzymatic assay, ß-glucan content was also measured in 18 primitive species of *Avena* to evaluate possible sources of germplasm for expanding the range of ß-glucan content currently available in domestic cultivars. A comparison of ß-glucan content with protein content, oil content and thousand kernel weight in domestic oats showed that these quality parameters are independent of ß-glucan concentration in oats. Differences in molecular structure of ß-glucan, as indicated by differences in molar ratios of trisaccharide:tetrasaccharide released during lichenase digestion, were observed among domestic and primitive oats.

Scanning microspectrofluorometry was used to map ß-glucan distribution in single kernels of oats: differences were observed within single kernels, and also among kernels from different cultivars of oats. All of the oats examined had high concentrations of ß-glucan adjacent to the germ. In oats with low total ß-glucan, high concentrations of ß-glucan were also observed around the periphery of the kernel, with smaller amounts in the central endosperm. As the total ß-glucan content of the samples increased, the high peripheral concentration of ß-glucan became less distinct. Microscopic examination suggests that the different distribution patterns are due to differences in cell wall thickness adjacent to the germ and around the periphery of the kernel, and also to differences in cell size and shape in the central endosperm.

A high ß-glucan (Marion) and a low ß-glucan oat cultivar (OA516-2) were selected for isolation and preliminary characterization of the endosperm cell walls, which are the major source of ß-glucan in the oat kernel. A method for isolating endosperm cell walls in wheat and barley was modified to accommodate the different chemical and physical characteristics of oats, and cell walls of both cultivars were isolated. Whole cell wall preparations were analyzed for monosaccharide composition, ß-glucan content, phenolic acid content, and amino acid profile. The differences in ß-glucan content that were observed in whole groats were not reflected in the composition of the isolated endosperm cell walls: preliminary characterization suggested that the cell walls of the two cultivars were very similar with respect to monosaccharide composition and ß-glucan content. Fractionation patterns after enzyme hydrolyses were also very similar for cell walls from both cultivars. It was therefore concluded that the differences in ß-glucan content that were observed in whole groats were not due to differences in the composition of isolated endosperm cell walls, but to variation in cell size and cell wall thickness in different areas of the groats.

to my father,
whose spirit still gives me encouragement

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And last but not least I would like to thank my mom and my family, who have been behind me all the way, and my husband, Michael, who jumped in in the middle with such patience and support.

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LIST OF ABBREVIATIONS

d.p.	degree of polymerization
FIA	flow injection analysis
GC	gas chromatography
GMA	glycol methacrylate
HPLC	high performance liquid chromatography
LDL	low density lipoprotein
MS	mass spectrometry
PAD	pulsed amperometric detection
RFI	relative fluorescence intensity
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TKW	thousand kernel weight

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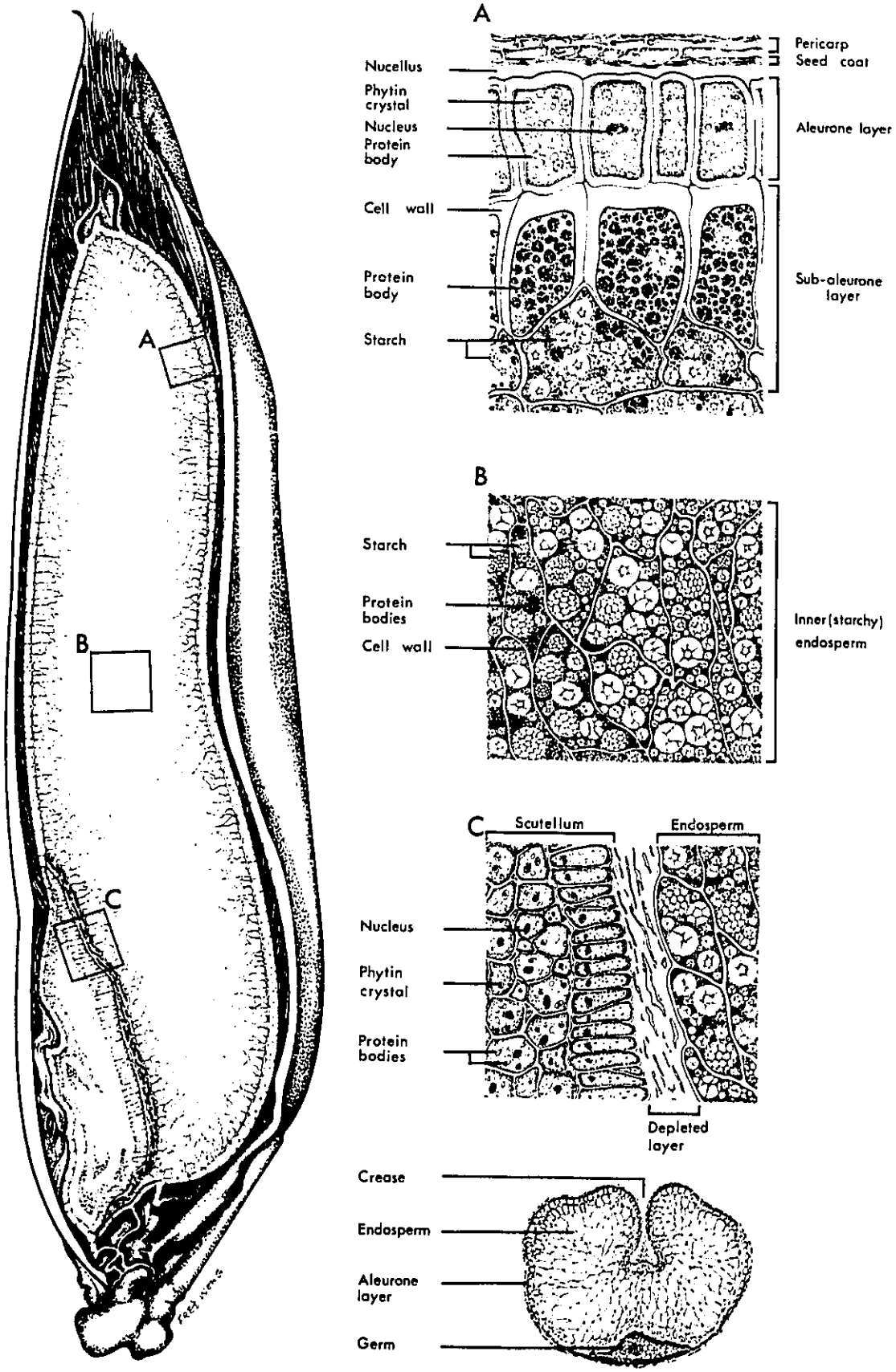
1. INTRODUCTION

Oat ß-glucans are linear cell wall polysaccharides composed of (1→4) and (1→3) linked ß-D-glucopyranose units in a ratio of between 2 and 3 to 1, depending on the tissue and the stage of maturity (Buchala and Wilkie 1971; Buchala and Meier 1973a; Yamamoto and Nevins 1978; Aspinall and Carpenter 1984; Wood 1984; Heims and Steinhart 1991). The mixed-linkage (1→3),(1→4)-ß-D-glucan (hereafter referred to as ß-glucan) in oats is part of a family of polysaccharides that are found almost exclusively in the Gramineae (Stinard and Nevins 1980). Of the grasses, the highest levels of ß-glucan are found in oats (*Avena sativa*) and barley (*Hordeum vulgare*), with the highest quantities being found in the seeds (McCleary and Glennie-Holmes 1985; Henry 1985; Fincher and Stone 1986; Henry 1987).

Figure 1 shows the anatomy of an oat kernel, and illustrates the structural features present in seeds of all members of the Gramineae (Fulcher 1986). Once the hull is removed, the kernel can be considered as three main components: the germ, the bran and the starchy endosperm. The germ, at the proximal end of the kernel, contains the embryo and the scutellum, shown in part of inset C of Fig. 1. The bran surrounds the starchy endosperm (B) and part of the germ, and consists of the pericarp, seed coat, nucellus and the aleurone layer. Although strictly speaking these tissues alone comprise the bran, in milling the aleurone usually separates from the grain with the first layer of cells from the starchy endosperm attached: the sub-aleurone layer. These tissues are shown in inset A, at the distal end of the kernel in Fig. 1. The third component, the starchy endosperm, makes up the largest proportion of the kernel. The major features of the starchy endosperm are shown in inset B, which is located in the central portion of the kernel in Fig. 1.

Figure 1. Diagram showing the anatomical components of a typical cereal grain, in this case, oats (*Avena sativa* L.). The area of the kernel including the germ (from the inset marked C to the lower tip of the kernel) is referred to as the proximal region of the kernel in the text. The inset marked B is in the central region of the kernel, and the inset marked A is in the distal region of the kernel. The cross-section at the lower right of the figure shows an area through the proximal region, containing some germ tissue. (From Fulcher 1986).


A
B
C
A
Pericarp
Seed coat
Nucellus
Phytin crystal
Nucleus
Protein body
Aleurone layer
Cell wall
Protein body
Sub-aleurone layer
Starch
B
Starch
Protein bodies
Cell wall
Inner (starchy) endosperm
C
Scutellum
Endosperm
Nucleus
Phytin crystal
Protein bodies
Depleted layer
Crease
Endosperm
Aleurone layer
Germ

Microscopic evidence indicates that the ß-glucan in oats and barley is found primarily in the cell walls of the starchy endosperm, with smaller amounts also present in the aleurone layer (Wood and Fulcher 1978; Fulcher and Wong 1980; Fulcher 1982; Fulcher and Wong 1982; Wood and Fulcher 1983; Fulcher 1986; Fincher and Stone 1986). In barley, the microscopic evidence has been confirmed by chemical characterization of isolated endosperm cell walls (Fincher 1975; Palmer 1975; Ballance and Manners 1978; Ahluwalia and Ellis 1985; Brunswick *et al* 1988). In oats, however, analyses have been performed on material that is ill-defined: either whole flour, or heterogeneous mixtures of cell walls derived from groats (oats with the hull removed: Wood *et al* 1977, 1978, 1991a; Selvendran and DuPont 1980; Aspinall and Carpenter 1984; Heims and Steinhart 1991). To date, analyzable quantities of pure endosperm cell wall have not been isolated from oats. Nevertheless, certain features of the ß-glucans (and other cell wall polysaccharides) in oats and barley have emerged, and these polymers have exhibited variation in quantity, structure, molecular weight, solubility, and distribution (Woodward and Fincher 1983; Fulcher 1986; Fincher and Stone 1986; Wood 1984, 1986; Henry 1986, 1987; Åman and Graham 1987a; Wood *et al* 1991a).

The fundamental curiosity inspired by this unique family of polymers is enhanced by more pragmatic concerns in the fields of nutrition and industrial application. Inclusion of soluble dietary fibre from a variety of sources has been reported to improve glucose and lipid metabolism. Consumption of soluble dietary fibre included in meals or as oral doses with simultaneous administration of glucose has been shown to reduce postprandial glycemia and insulin responses. These effects have been noted with oat gum (which is primarily ß-glucan: Wood 1984; Siebert 1987) as well as with guar gum and other soluble dietary fibres (Jenkins *et al* 1980a,b; Krotkiewski 1984; Wood *et al* 1989,

1990; Braaten *et al* 1991; Pastors *et al* 1991). Jenkins *et al* (1980b) reported that prolonged consumption of guar reduced urinary glucose losses, and lowered insulin requirements in diabetics. In addition to improvements in glucose metabolism, current evidence indicates that the inclusion of oat products, or soluble dietary fibre from other sources, can significantly lower serum cholesterol levels, particularly in hypercholesterolemic individuals. In spite of a recent report to the contrary (Swain *et al* 1990), it appears that a diet containing oat ß-glucan (in a natural oat product, or as an additive) results in reduced serum total cholesterol levels, as well as reduced levels of low density lipoprotein (LDL) cholesterol (Kirby *et al* 1981; Anderson *et al* 1984; Anderson and Chen 1986; Anderson and Gustafson 1988; Anderson *et al* 1990; Demark-Wahnefried *et al* 1990; Wood *et al* 1989; Davidson *et al* 1991; Van Horn *et al* 1991). LDL-cholesterol is an important factor in the development of coronary heart disease (Kannel *et al* 1979; Gotto 1986). Other benefits of soluble fibre consumption may include increased satiety and weight normalization (Krotkiewski 1984). If sources with sufficiently high levels of ß-glucan were available, oats and oat products have the potential to provide highly palatable dietary adjuncts which would be useful in the management of mild diabetes or hypercholesterolemia.

In contrast to the reported clinical benefits in human nutrition, high levels of ß-glucan in poultry feed have been reported to have deleterious effects. Increasing the ß-glucan content of poultry diets by inclusion of either oats or barley has been shown to result in low feed conversion efficiencies, and lower, slower weight gains than other diets (Potter *et al* 1965; Hesselman *et al* 1982; Cave *et al* 1990). Additional supplementation of a ß-glucan-containing diet with ß-glucan-degrading enzymes resulted in increased weight gains and feed conversions, indicating that the deleterious effects were caused

by ß-glucan (Hesselman *et al* 1981, 1982; Cave *et al* 1990).

Although the use of oat gum for its functional properties (as opposed to therapeutic merits) remains largely unexplored by the food industry to date, the information available suggests that oat ß-glucan would be well able to compete with other plant gums currently in use (Wood *et al* 1978; Wood 1984; Autio *et al* 1987). In addition to direct enrichment to supplement a product with soluble dietary fibre, water soluble plant gums can serve in food products as processing aids, thickening agents, adhesive coatings and to improve moisture retention of baked goods (Andon 1987; Anderson and Andon 1988).

Manufacturers in the cereal industry have invested large amounts of money to improve oat quality parameters such as oil content, protein content, disease resistance and yield. With the upsurge in interest in the reported clinical benefits of ß-glucan in the diet, ß-glucan content has been added to the list of oat quality parameters that require more precise definition and possibly improvement as well.

A survey of the literature indicates that there are differing levels of ß-glucan in different varieties of oats and barley, and that environmental effects may also influence ß-glucan levels (Aastrup 1979; Coles 1979; Gill *et al* 1982; Molina-Cano and Conde 1982; Hesselman and Thomke 1982; Bourne and Wheeler 1984; Åman 1986; Henry 1986; Palmer and MacKenzie 1986; Lehtonen and Aikasalo 1987; Truelsen 1987; Åman and Graham 1987b; Wood *et al* 1991a). These studies beget questions requiring more detailed answers. What is the range of ß-glucan content currently available in domestic oat cultivars? What is the influence of environment on ß-glucan levels in oats, and how strong is it? Is there an association between ß-glucan content and other oat quality parameters? Are there sources available, for example in primitive species of *Avena*, which

could be used to modify or enhance ß-glucan content in domestic cultivars? Are there differences in structure and/or distribution of ß-glucan in different varieties of oat that could affect functional properties or end-uses in those varieties? Is the range of ß-glucan content different in the endosperm cell walls of cultivars of oats with different levels of total ß-glucan, and if so, how does the overall cell wall composition differ in these cultivars? These are the kinds of questions that have driven the design of the experiments in this thesis.

Hypotheses

The questions posed in the introduction can be briefly summarized in two hypotheses:

1. The ß-glucan content of oat germplasm varies based on the genotype of the oats, and the environmental conditions of growth.
2. The composition of the endosperm cell walls of different cultivars varies with the total ß-glucan content of those cultivars.

Rationale

An accurate, reliable method for measurement of oat ß-glucan was a prerequisite for the studies proposed. The majority of methods reported in the literature for the measurement of cereal ß-glucans were developed for barley (see review by Jorgensen and Aastrup 1988a). Assessment of the advantages and disadvantages of the various methods led to the choice of an enzymatic assay (McCleary and Glennie-Holmes 1985) which, in addition to a high degree of specificity and reproducibility, possessed the added advantage of commercial availability in a kit containing enzymes of certified purity.

After confirming the specificity and purity of the (1→3),(1→4)-ß-D-glucan 4-glucanohydrolase (lichenase) in the kit, the assay, which was developed to measure barley ß-glucan, was modified to measure ß-glucan in oats by adjusting the lichenase digestion time and the recommended grinding protocol. Results from the modified enzymatic assay were compared with the results obtained by flow injection analysis (FIA), which has been reported to give results comparable to those obtained using enzymatic methods (Åman and Graham 1987b; Jorgensen 1988; Jorgensen and Aastrup 1988b; Sendra *et al* 1989; Switala *et al* 1989; Anderson 1990). Although the correlation between the two methods was found to be quite high in the present study (r=0.90), it was concluded that the enzymatic assay afforded a more accurate estimation of the total ß-glucan in oats.

To assess the range of genetic and environmental variation in oat ß-glucan content, the modified enzymatic assay was used to measure the ß-glucan content in a number of domestic cultivars and breeder's lines of Canadian oats grown in 5 locations in 3 growing seasons. These samples were obtained from the Eastern Co-operative Oat Test (Agriculture Canada), which also supplied additional information regarding the more traditional quality parameters of oats, such as protein and lipid content. The extent of variation within the genus *Avena* was also explored, and experiments were performed to examine the possibility of variation in the fine structure of ß-glucan, both within *Avena sativa* and among different species in the genus *Avena*. And finally, to address both the biological question of the role of ß-glucan in the seed and the economic practicalities of ß-glucan in specific oat products (e.g. oat bran), variation in distribution of ß-glucan within single kernels, and among kernels of different cultivars of domestic oats, was studied.

In order to begin to truly understand the role of ß-glucan in the kernel, it is necessary to look more closely at the component of the seed where the ß-glucan is found: the endosperm cell wall. Cultivars with high and low ß-glucan contents were selected from the samples analyzed for isolation and characterization of endosperm cell walls. By choosing cultivars with differing levels of ß-glucan, a better understanding of its interaction with other polymers and of its function within the cell wall was envisaged.

For each sample, it was necessary to isolate a sufficient quantity of pure endosperm cell walls for analysis. The method used by Mares and Stone (1973a) to isolate wheat endosperm cell walls was selected as a starting point. In this method, the cell wall isolation is performed in 70% ethanol, thus preventing the loss of water-soluble ß-glucan noted in the aqueous procedure of Selvendran and DuPont (1980). Nevertheless, the problem of coprecipitation of cytoplasmic compounds (Selvendran and DuPont 1980) had to be addressed, as well as new problems arising from chemical and morphological differences between wheat and oats which were encountered as the isolation progressed.

A traditional fractionation of cell wall polysaccharides based on sequential extractions in increasingly strong solvents often results in non-specific disruption of a variety of bonds in the walls, and incomplete and frequently overlapping cell wall fractions (Dey and Brinson 1984; Kato and Nevins 1984). The selective dissociation of walls by enzymatic hydrolysis, under gentler conditions than the traditional procedures, has the potential to release fragments more characteristic of the native polysaccharides than those derived from treatment with chemical reagents (Kato and Nevins 1984), and was considered a better approach for characterization of the oat endosperm cell wall. After determination of monosaccharide composition to provide an estimate of the types and

relative proportions of the polysaccharides present, the walls were fractionated by sequential hot water extraction, enyzmatic degradation of the major polysaccharides indicated (ß-glucan and arabinoxylan), and finally, determination of the composition of the insoluble, undegraded residue by acid hydrolysis. ß-Glucan in both the hot water extract, and in the water-insoluble residue was hydrolyzed using lichenase. For degradation of the arabinoxylan, enzyme was harvested from an *Escherichia coli* clone with a plasmid carrying the endoxylanase gene from *Bacteroides succinogenes*. The cloned enzyme was chosen to avoid contamination with non-specific activities that is often found in commercial enzyme preparations.

The results of these studies improve the current definition of oat ß-glucan and some of the types and sources of variation; and in addition, provide new insight into the biochemistry and structure of the oat endosperm cell wall.

2. REVIEW OF PERTINENT LITERATURE

2.1 Plant Cell Walls

The highest concentrations of ß-glucans are found in the endosperm cell walls of oats and barley. In cereal grains, the walls of the aleurone layer, the endosperm and the scutellum are characteristic of primary cell walls (Fincher and Stone 1986), which are deposited during the expansion or elongation stage of plant development (Dey and Brinson 1984; McNeil *et al* 1984; Fincher and Stone 1986; Bacic *et al* 1988).

The primary cell walls of plants are composed mainly of cellulose microfibrils embedded in a matrix of other polysaccharides. In addition to the polysaccharide components, smaller amounts of protein and phenolic acids are also present (McNeil *et al* 1984; Fincher and Stone 1986). The primary walls of different plant taxa vary in composition with respect to all of the major components (Bacic *et al* 1988).

Within the angiosperms (flowering plants), distinct differences between the walls of monocotyledonous plants (monocots: represented primarily by the grasses) and those of dicotyledonous plants (dicots) have been documented. The range of cellulose contents for primary cell walls of dicots is 9-40%, and for the grasses, 2-40% (Bacic *et al* 1988) depending on species and tissue. The main matrix polysaccharides of dicots are pectic polysaccharides and xyloglucans: 34% and 20% respectively (in suspension cultured sycamore cells: Darvill *et al* 1980; McNeil *et al* 1984). These polysaccharides are only minor components in the grasses, where ß-glucans and arabinoxylans are the major matrix polysaccharides (Wada and Ray 1978; Labavitch and Ray 1978; Darvill *et al* 1980; Fincher and Stone 1978; Bacic *et al* 1988). Variation in the cell wall glycoproteins, which can be up to 10% of the wall of both groups, has also been

demonstrated. In dicots, the structural proteins are characteristically rich in hydroxyproline, and virtually all of the hydroxyproline residues are glycoslyated with mono-, di-, tri- or tetra-arabinosides; the tetra-arabinoside predominates (Lamport and Miller 1971). In vegetative tissues of the grasses, approximately one third of the hydroxyproline residues in the cell wall proteins are glycoslyated; here the tri-arabinoside predominates (Lamport and Miller 1971).

2.1.1 Cell walls of oat and barley endosperm

Attempts to determine the composition of oat cell walls have yielded conflicting data. A mixture of aleurone and endosperm cell walls from oats (isolated in aqueous media, with loss of up to 10% of soluble ß-glucans) contained 8% cellulose (Selvendran and DuPont 1980). A similarly heterogeneous preparation from oat bran, however, was reported to contain only traces of cellulose (less than 0.5%, Aspinall and Carpenter 1984). In the oat bran sample, Aspinall and Carpenter (1984) estimated greater than 14% ß-glucan, while Selvendran and DuPont (1980) were unable to accurately determine the ß-glucan content of their preparation of aleurone and endosperm cell walls due to losses incurred during isolation of the walls, and contamination with starch. Estimation of the polysaccharide content of the oat bran cell walls was also complicated by excessive (~50%) contamination with starch (Aspinall and Carpenter 1984). The arabinoxylan content of oat cell wall material was calculated to be approximately 39% from the data presented by Selvendran and DuPont (1980), while Aspinall and Carpenter (1984) reported only about a tenth of that amount (>3%). Both galactose and uronic acid were detected in the oat cell wall preparation of Selvendran and DuPont (1980), but only galactose was reported by Aspinall and Carpenter (1984). Oat cell walls have been

reported to contain 6.1% protein (Selvendran and DuPont 1980) and 0.75-0.81% of total phenolic acids (ferulic and *p*-coumaric: Selvendran and DuPont 1980; Hartley 1987).

In contrast to the somewhat ambiguous information available for oat cell walls, which is complicated by analyses of cell wall mixtures rather than cell walls from a single tissue, the cell wall composition of barley endosperm has been well characterized (Fincher 1975; Palmer 1975; Forrest and Wainwright 1977; Ballance and Manners 1978; Ahluwalia and Ellis 1985; Ahluwalia and Fry 1986). Analysis of the monosaccharide composition of the walls shows 74-79% glucose, 10-13% xylose, 9-11% arabinose, and 2-5% mannose (Fincher 1975; Forrest and Wainwright 1977; Ballance and Manners 1978; Ahluwalia and Ellis 1985). Galactose has not been detected and only small amounts of uronic acid are present (Fincher 1975), suggesting that pectic polysaccharides are not a significant component of the endosperm wall. Protein was determined as less than 5% of the whole walls; no hydroxyproline was detected (Fincher 1975; Ballance and Manners 1978; Ahluwalia and Ellis 1985). Ahluwalia and Fry (1986) reported 0.06% ferulic acid in endosperm cell walls of several varieties of barley. Up to 49% of the whole wall is soluble in water, depending on the temperature of extraction (Fincher 1975, Forrest and Wainwright 1977; Ballance and Manners 1978).

ß-Glucan accounted for the majority of the glucose in barley endosperm cell walls, representing 70-75% of the whole wall (Fincher 1975, Forrest and Wainwright 1977; Ballance and Manners 1978). Although a microfibrillar network was visible after successive extractions with water and alkali, only small amounts of cellulose were reported. Ballance and Manners (1978) suggested that the microfibrillar phase probably consisted of cellulose (less than 1%) together with tightly bound arabinoxylan and glucomannan. The arabinoxylan in the cell walls carried virtually all of the ferulic acid, and

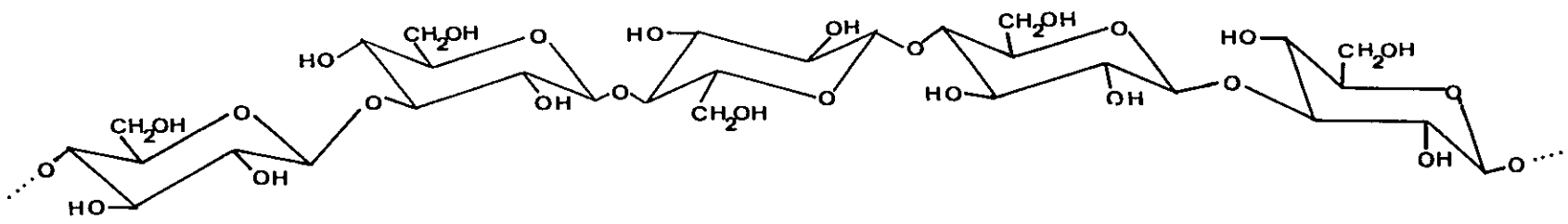
appeared to be esterified to the polysaccharide via the arabinose residues (Ahluwalia and Fry 1986).

2.2 ß-Glucans in Cereals

2.2.1 Occurrence

Mixed-linkage ß-glucans (Fig. 2) belong to a family of unbranched polysaccharides containing (1→4)- and (1→3)-linked ß-D-glucopyranosyl units in varying proportions (Wood 1986). With the exception of some lichens (Peat *et al* 1957; Takeda *et al* 1972; Nishikawa *et al* 1974), ß-glucan is restricted to the Graminea. ß-Glucans have been identified in both reproductive (grain) and non-reproductive tissues of oats, barley, wheat, rye, corn, sorghum, rice and triticale (Morris 1942; Preece and MacKenzie 1952a,b; Preece and Hobkirk 1953; Buchala and Wilkie 1970, 1971; Buchala and Meier 1973a,b; Buchala and Wilkie 1973a,b; Woolard *et al* 1976; Wood *et al* 1977; Anderson *et al* 1978; Nevins *et al* 1978; Shibuya and Misaki 1978; Bacic and Stone 1980; Madacsi *et al* 1983; Henry 1987; Shibuya *et al* 1985). In addition, ß-glucan has been reported in all of the major sub-families of the Gramineae, but not in representatives of the other major families of monocotyledonous plants (Stinard and Nevins 1980). ß-glucan was reported to occur transiently in immature mung bean (Buchala and Franz 1974), but this has not been repeated, and remains the only evidence of the presence of mixed-linkage ß-glucan in dicotyledonous plants, or indeed, in any of the angiosperms outside of the grasses. *In vitro* synthesis of a mixed ß-(1→3),(1→4)-glucan was reported in a membrane fraction isolated from pine (a gymnosperm) seedlings. This synthesis was dependent upon the precursor supplied (Dalessandro *et al* 1988), and natural formation of the polysaccharide in pine has not been reported.

Figure 2. Diagram illustrating the chemical structure of cereal ß-glucans.


HO
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O

2.2.2 Physicochemical properties

Although the general structure of all of the cereal ß-glucans (Fig. 2) is similar (a linear molecule composed mainly of cellotriosyl and cellotetraosyl units joined by single (1→3) linkages: Wood 1984), differences in physical and chemical properties such as molecular weight, and ratios and distribution of (1→3) and (1→4) linkages in the polysaccharide have been reported. These differences have led to the widely held view that the cereal ß-glucans represent a large family of polysaccharides of varying molecular size and fine structure (Woodward and Fincher 1983; Wood 1986). The differences reported depend on species, tissue, method of extraction, and method of determination of a particular characteristic. The last two factors may lead to a perception of differences in data presented in the literature that are at least partially artefactual. Indeed, reported differences in viscosity are particularly difficult to evaluate (because of differing conditions or unreported methods of estimation, Wood 1984), and will not be discussed in this review. Significant differences in ß-glucan solubility have been noted between oats and barley: barley ß-glucan (38-69% soluble) is, in general, less soluble than oat ß-glucan (65-90% soluble) (Åman and Graham 1987a). Such solubility differences could reflect other differences in chemical and physical properties, or simply the choice of varieties.

Molecular weight: More information on molecular weight is available for barley ß-glucans than for oat ß-glucans. Woodward and Fincher (1983) summarized the molecular weights determined by a number of workers for various fractions of water-soluble ß-glucan from barley grains, and the range was very broad, from $2x10^4$ to $4x10^7$. Forrest and Wainwright (1977) reported a decrease in molecular weight from $34x10^6$ to $1.3x10^6$ on treatment of water- or alkali-extracted barley ß-glucan with either thermolysin or hydrazine, suggesting

that several ß-glucan molecules are linked to a protein core to form a larger molecule.

Most of the available data for oat ß-glucan, while sparse, suggests smaller molecular weights than those reported for barley. Values in the range of 2.68-6.3x10^4 have been reported (Acker *et al* 1955; Podrazky 1964), as well as higher values: 1.6-2.7x10^5 (Smith and Montgomery 1959; Vårum and Smidsrod 1988). However, results reported by Wood (1986) from gel filtration studies suggest that the molecular weight of oat ß-glucan is actually much higher, exceeding that of the high molecular weight standard Blue Dextran which has a molecular weight of approximately 2x10^6.

Fine structure of cereal ß-glucan: The (1→4) and (1→3) linkages in a ß-glucan can be estimated by consumption of periodate, Smith degradation (periodate oxidation followed by mild acid hydrolysis), or methylation analysis (Aspinall 1970). The latter two methods are more commonly applied, as they allow direct estimation of (1→3) as well as (1→4) linkages.

In non-endospermic tissues of oats (Buchala and Wilkie 1971; Buchala and Meier 1973a), barley (Buchala and Wilkie 1973a), wheat (Buchala and Wilkie 1973b) and also bamboo (Wilkie and Woo 1976), the ratio of (1→4) to (1→3) linkages in hemicellulosic ß-glucan increased with increasing tissue maturity. In endospermic tissues of oats and barley, the ratio of (1→4) to (1→3) linkages is between 2 and 3 to 1 (Igarishi and Sakurai 1965, 1966; Woodward *et al* 1983a,b; Wood 1984; Aspinall and Carpenter 1984; Heims and Steinhart 1991), although lower values have been reported (Smith and Montgomery 1959). A ratio of 1.5:1 was reported for sorghum endosperm (Woolard *et al* 1976), and a very unusual ratio of approximately 1:6 was reported in rice endosperm (Shibuya and Misaki 1978).

The presence of contiguous (1→3) linkages was reported in some of the early studies of endospermic tissues of both oats (Smith and Montgomery 1959; Goldstein *et al* 1965) and barley (Igarishi and Sakurai 1966; Fleming and Manners 1966; Bathgate and Dalgleish 1975; Fleming and Kawakami 1977). However, the Smith degradation technique used to demonstrate double or multiple (1→3) linkages is open to misinterpretation due to artefacts: incomplete oxidation may lead to misidentification of (1→3) linkages. Lichenan, which is structurally similar to ß-glucan, is much more resistant to periodate oxidation than α-linked amylose (Aalmo *et al* 1978). Woodward *et al* (1983b) have shown that the time required for complete hydrolysis of the oxidized polysaccharide is considerably longer than times reported in earlier studies, so that incomplete hydrolysis may also have contributed to misidentification of (1→3)-linked oligomers. Early methylation techniques were prolonged and inefficient, often requiring months to achieve near-complete methylation. With the introduction of the Hakamori method (Hakamori 1964), procedures for methylation were much improved, and complete methylation could be achieved in a fraction of the time required by the older methods. More recent studies of both oat (Aspinall and Carpenter 1984; Vårum and Smidsrod 1988; Wood *et al* 1991a) and barley (Woodward *et al* 1983b; Woodward *et al* 1988; Edney *et al* 1991) ß-glucans using more up-to-date methods for Smith degradation and methylation have failed to demonstrate the presence of contiguous (1→3) linkages.

While linkage analysis provides essential information on the basic structure of ß-glucans, structural variation is more readily demonstrated by examination of the oligosaccharides generated by lichenase digestion of the polysaccharide. Tri- and tetrasaccharides (3-O-cellobiosyl-D-glucose and 3-O-cellotriosyl-D-glucose respectively) make up approximately 90% of the total ß-glucan of barley and oats, with the remainder

of the molecule being composed of longer stretches of (1→4) linkages up to a degree of polymerization (d.p.) of 9 (Woodward *et al* 1983b; Wood *et al* 1991a). Methylation analyses revealed relatively small differences in the percentage of (1→4) linkages between oat and barley ß-glucan (71%) and lichenan (67%), but the ratios of tri- and tetrasaccharides released by lichenase digestion were 2.0 and 3.1 for oat and barley ß-glucans respectively, and 20.2 for lichenan (Wood *et al* 1991a).

Structural variation as revealed by differences in tri-: tetrasaccharide ratios have been shown in non-endospermic ß-glucans from a wide selection of grasses (Nevins *et al* 1977; Nevins *et al* 1978; Stinard and Nevins 1980; Kato and Nevins 1986). In endospermic ß-glucans, differences are also evident, both between species, and among different cultivars within a species (Wood *et al* 1991a). The range observed was widest for wheat (3.04-3.84) and narrowest in oats (2.09-2.25), while the range for barley was intermediate (2.93-3.41).

Conformation: Studies on barley ß-glucan suggest that it assumes a worm-like chain conformation in solution, with flexibility arising from the introduction of a (1→3)-linkage into a stretch of (1→4)-linkages (Woodward *et al* 1983a). Wood (1984) presented a series of photographs of space-filling models comparing cellulose (composed entirely of (1→4)-linked glucose), which has a flat, ribbon-like conformation, and curdlan (composed entirely of (1→3)-linked glucose) which adopts a hollow, spring-like conformation. Both of these polymers are insoluble in water, and require acid- or alkaline extraction (Wood 1984). Cereal ß-glucan, which contains both types of linkages, is very soluble in water, and adopts an intermediate type of conformation, which, while having greater flexibility than either cellulose or curdlan, nevertheless presents cellulose-like surfaces (Woodward

et al 1983b; Wood 1984). These surfaces are thought to hydrogen-bond together to form "junction zones" (Fincher and Stone 1986), and could play an important role in orienting the molecules in the matrix of the cell wall and in gel formation.

2.2.3 Extraction and isolation of endospermic ß-glucan

The common nomenclature of the cereal ß-glucans and arabinoxylans refers to gums and hemicelluloses: gums being that proportion of the ß-glucan and arabinoxylan which is extractable in water, and hemicelluloses (the water insoluble, non-cellulosic ß-glucans and arabinoxylans) requiring alkali for solubilization. Nevertheless, the basic chemical structure in both gums and hemicelluloses can be the same (Bathgate and Dalgleish 1975). A large portion of the endospermic ß-glucan in some cereals, particularly oats and barley, is water soluble. In other cereals, with lower levels of ß-glucan such as wheat (Bacic and Stone 1980; Beresford and Stone 1983), rice (Shibuya and Misaki 1978), sorghum (Woolard *et al* 1976; Madacsi *et al* 1978) and rye-grass (Anderson and Stone 1978) the polymer requires stronger reagents for extraction. Quantitative extraction and isolation of ß-glucans from cereal grains is difficult, owing to problems in complete extraction of the polysaccharide free from contamination with starch, pentosan and protein (Wood 1986).

Many of the studies from which much of our present knowledge of oat and barley ß-glucan is derived were performed on fractions soluble in water at various temperatures, for both ease and gentleness of extraction (Morris 1942; Preece and MacKenzie 1952a,b; Preece and Hobkirk 1953; Igarishi and Sakurai 1965, 1966; Fleming and Kawakami 1977; Forrest and Wainwright 1977; Staudte *et al* 1983; Woodward *et al* 1983a,b, 1988; Ahluwalia and Ellis 1985). The amount of water soluble glucan in extracts increases with

increasing temperature of extraction (Fleming and Kawakami 1977; Ahluwalia and Ellis 1985). The dependence of ß-glucan yield on extraction temperature also holds for extraction in alkaline buffers (Wood *et al* 1978; Vårum and Smidsrod 1988). Wood *et al* (1978) reported that in addition to temperature, extraction of ß-glucan from oats in alkaline buffer was affected by flour particle size, pH, and ionic strength of the media.

Further extraction of the polymer, either for purposes of quantitation or for chemical studies, requires stronger and often lengthy extraction methods. Carbonate buffer at pH 10 was used by several workers (Wood *et al* 1977, 1978; Madacsi *et al* 1978; Prentice *et al* 1980), although the completeness of extraction in these studies is unclear. It has been pointed out that while it is relatively simple to monitor and establish the limit of a particular extraction method, often little information is gained about residual ß-glucan in the sample (Wood 1984). Hydrazine, which cleaves peptide bonds but not glycosidic linkages, has been reported to achieve complete solubilization of ß-glucan in barley after lengthy extraction (40 hrs, 104°C)(Martin and Bamforth 1981). Indeed, Bamforth (1982) claims that only hydrazinolysis guarantees complete extraction of hemicellulose from barley. However, equivalent results were later reported with 4% NaOH. Sodium hydroxide presents much less potential hazard to the worker, but a significant potential for alkaline degradation of the ß-glucan, in the rather long extraction period (24 hrs: Palmer and MacKenzie 1986). A low concentration of hot perchloric acid for short periods of time has also been used to effect complete solubilization of ß-glucan in barley (Ahluwalia and Ellis 1984; Jorgensen and Aastrup 1988b; Anderson 1990; Edney *et al* 1991). Ahluwalia and Ellis (1984) reported that extraction was complete after 3 minutes in 50 mM perchloric acid at 96°C, and that further extraction over a period of 10 minutes did not increase the yield. This is in contrast to the data reported by Jorgensen and

Aastrup (1988b), who showed that, after an initial extraction in boiling water, an increase in ß-glucan yield up to 10 minutes extraction in dilute acid was followed by a decrease in yield with longer extraction time due to acid hydrolysis of the polymer. Dilute sulfuric acid (75 mM) or HCl (100 mM) was found to be as effective as 100 mM perchloric acid, and the less hazardous sulfuric acid was routinely used for extraction (Jorgensen and Aastrup 1988b). Analysis of the residue by an enzymatic method showed that less than 0.2% glucan remained after extraction. Voragen *et al* (1987) reported virtually complete solubilization of barley endosperm cell walls using 4-methylmorpholine-N-oxide, with no degradation of the major polymers (ß-glucan and arabinoxylan) present.

Isolation and purification of ß-glucan from other co-extracted components involves adjustment of the pH to around 4 to precipitate proteins, and then selective precipitation of the ß-glucan from other polysaccharides present in the extract (Wood 1984, 1986). Selective precipitation has been reported using 20% ammonium sulfate (Preece and MacKenzie 1952a; Preece and Hobkirk 1953), complexing with copper (Madacsi *et al* 1983), and complexing with specific dyes (Wood 1985). Alternatively, the ß-glucan in the extract can be digested using a specific enzyme (lichenase), which cleaves the polysaccharide to characteristic oligosaccharides (Anderson *et al* 1978; McCleary and Glennie-Holmes 1985). The pattern of oligosaccharide products can then be used to obtain both quantitative and structural information on the ß-glucan in a particular sample. Because of the high degree of specificity of the enzyme, it can be used directly on a sample without prior extraction. For this reason enzymatic studies are particularly attractive. They thus avoid some of the pitfalls of extraction methods cautioned against by Wilkie (1985a): the selection of a small and not necessarily representative fraction from the total ß-glucan.

2.2.4 Direct methods for determination of ß-glucan

Because of the importance of ß-glucan in the brewing industry (for reviews see Bamforth 1982; McCleary 1986), the majority of methods to measure it, both directly and indirectly, have been developed for barley, malt, wort and beer (see review by Jorgensen and Aastrup 1988a). A number of direct methods of ß-glucan determination have been proposed which involve an extraction step and precipitation of the extracted components from solution by the addition of a particular reagent or solvent. The material precipitated from the resulting extract is then hydrolyzed either with acid or enzymes. Precipitation techniques restrict by size the ß-glucan which is measured, and rely heavily on precise experimental conditions (Bamforth 1982). The assumption in these methods is that only starch and ß-glucan are solubilized in the extraction. Other glucans, such as xyloglucan or ß-(1→3)-D-glucan, could also conceivably contribute to the total glucan if present (Wood 1984).

Wood *et al* (1977) introduced the specific determination of total glucan and starch in alkaline extracts from oats (carbonate buffer, pH 10, 45°C). The pH of the extract was adjusted to 4.5 to precipitate proteins, followed by alcohol precipitation of high molecular weight polymers. The starch in the precipitated gum was hydrolyzed to glucose by amyloglucosidase, and the glucose measured using glucose oxidase. Total glucan in the gum was determined by cysteine-sulfuric acid reagent, and ß-glucan determined as the difference between the total glucose and glucose derived from starch. A small amount of pentosan was also determined to be present in the gum, but the cysteine-sulfuric acid reagent was considered to be specific for glucose at the wavelength used (428 nm). Although the extraction methods used are stronger than those previously described, the complete extraction of ß-glucan by this method is by no means certain (Bamforth 1982).

A major advantage of this method, however, is the direct determination of glucose in the extract; no calibration with another method is required.

Madacsi *et al* (1983) extracted gum from sorghum using a method similar to Wood *et al* (1977), but selectively precipitated ß-glucan with alkaline copper sulfate. This reagent contains the anion $Cu(OH)_4^{2-}$ which, when tested on solutions containing ß-glucan (lichenan) and soluble starch, formed a precipitate only with lichenan. The ß-glucan in the precipitate was analyzed by the phenol-sulfuric acid method for carbohydrate. As with all extraction procedures, the major drawback of this method is the uncertainty regarding the completeness of extraction.

Alkaline extraction conditions were also used by Palmer and MacKenzie (1986), who compared the efficacy of extraction with 4% NaOH for 24 hrs with that of hydrazine extraction. In both methods, the extracts were neutralized and dialyzed extensively against sodium acetate, and the ß-glucan in the dialyzate determined using ß-glucanases from *Trichoderma reesei*. it was concluded that 4% NaOH was as effective as hydrazine in extracting ß-glucan, and in fact preferable, with only 8% lost during dialysis, as compared to a 12% loss with hydrazine. The completeness of extraction of ß-glucan is not certain for this method. In addition, even at room temperature, it is highly likely that over the course of 24 hours, alkaline degradation of the ß-glucan would occur, which in addition to the losses during dialysis, may have obscured differences in the ß-glucan content of the barleys examined.

A number of direct methods for the measurement of ß-glucan utilize dyes that bind specifically to the ß-glucan. The fluorescent dye Calcofluor has long been recognized histochemically as a useful marker for plant cell walls (Hughes and McCully 1975). When solution studies were undertaken to investigate the specificity of the interaction of the dye

and the plant cell wall, the affinity of Calcofluor for polysaccharides containing ß-(1→4)-linked glucopyranosyl units became apparent (Maeda and Ishida 1967; Wood and Fulcher 1978; Wood 1980a,b; Wood 1982; Wood and Fulcher 1983). In the seeds of oats and barley, this selectivity translates to a specificity for the mixed linkage ß-glucans, as these are the major polysaccharides containing ß-(1→4)-linked glucose found in the seeds of these cereals. A number of assays have been developed to exploit this.

Calcofluor has been used to precipitate ß-glucan specifically from extracts of ground oats (Wood and Weisz 1984). Following acid hydrolysis of the dye-glucan complex, glucose can be determined by HPLC, or other methods. As with all extraction methods, the completeness of extraction remains a problem. Because the glucose from the hydrolyzed precipitate is determined directly, however, no calibration with another method is required. Subtraction of a starch component is unnecessary, as the dye does not interact with starch (Maeda and Ishida 1967, Wood 1980a).

The increase in relative fluorescence intensity (RFI) observed when Calcofluor complexes with cereal ß-glucan (Wood and Fulcher 1978; Wood 1980a,b; Wood 1982, Wood and Fulcher 1983; Wood 1985) has also been exploited in methods to measure ß-glucan. Complex formation occurs in solution (where precipitation is assisted by the addition of ethanol), or in solid flour samples (Jensen and Aastrup 1981). The resulting dye-polysaccharide complexes are washed extensively with ethanol to remove uncomplexed dye, suspended in glycerol, and the increase in RFI measured at 420 nm (excitation at 365 nm). The accuracy of this method was reported to be lower for flour samples than for extracts (Jensen and Aastrup 1981).

The dye Congo Red also shows specificity for ß-glucans. Complexing of ß-glucan with the dye was accompanied by a bathochromic shift in the absorption spectrum of

Congo Red (Wood and Fulcher 1978; Wood 1980; Wood 1982; Wood and Fulcher 1983), as well as a large increase in RFI at 590 nm (excitation at 546 nm). Quantitation of ß-glucan in solutions or extracts has also been reported using absorbance measurements of the Congo Red-polysaccharide complex at 550 nm, although complex formation was reported to be quite sensitive to the molecular weight of the ß-glucan (Anderson 1990).

The most successful adaptation of dye-complexing for routine ß-glucan measurement has been the development of Flow Injection Analysis (FIA) systems. A number of such systems, which measure the increase in RFI when ß-glucan in soluble extracts complexes with Calcofluor in buffered solution, have been reported (Jorgensen 1988; Jorgensen and Aastrup 1988b; Sendra *et al* 1989; Switala *et al* 1989; Anderson 1990; Manzanares *et al* 1991) with only minor differences between them.

As with all extraction methods for measurement of ß-glucan in cereal grains, the accuracy of the method is dependent upon complete extraction of the glucan. A one hour extraction in boiling water, followed by a mild acid extraction for ten minutes (also at 100°C) has been reported to completely extract all ß-glucan from barley flour (Jorgensen and Aastrup 1988b). The ß-glucan content of the freeze-dried residue from such an extraction was enzymatically determined to be less than 0.2% (Jorgensen and Aastrup 1988b). The extract was introduced into the FIA system where it was mixed with a buffered solution of Calcofluor and the resulting increase in RFI upon complexing of the dye with ß-glucan was measured. The system was calibrated with ß-glucan standards of known purity, with a fresh standard curve being run every day. As with Congo Red, formation of the Calcofluor-polysaccharide complex is somewhat sensitive to the molecular weight of the ß-glucan (Foldager and Jorgensen 1984; Anderson 1990; Manzanares *et al* 1991). However, FIA measurement of extracted ß-glucan by complexing

with Calcofluor has been reported to be in good agreement with enzymatic methods (Åman and Graham 1987b; Jorgensen 1988; Jorgensen and Aastrup 1988b; Aastrup 1988; Switala *et al* 1989) of ß-glucan determination.

A number of methods have been developed using specific enzymes to hydrolyze ß-glucan, either in extracts from cereal grains (Prentice *et al* 1980; Martin and Bamforth 1981; Ahluwalia and Ellis 1984) or directly in the ground material itself (Anderson *et al* 1978; Henry 1984; McCleary and Glennie-Holmes 1985; Åman and Hesselman 1985; Henry and Blakeney 1986,1988; Åman and Graham 1987b). The success of these methods is entirely dependent on the purity of the enzymes used. Ideally, the enzyme preparation should contain a single enzyme, or a limited combination of enzymes, so that only glucose from ß-glucan is released and measured. Fungal enzyme preparations are, almost without exception, a complex mixture containing a wide variety of hydrolytic activities. Contamination with starch-degrading activities is a particular problem, owing to the abundance of this polysaccharide in cereal grains. In the clean-up of fungal preparations reported, there is considerable variation in the pH and temperature conditions used to inactivate glucoamylases, and frequently levels of other interfering activities have not been adequately monitored (McCleary and Glennie-Holmes 1985). Bacterial enzyme preparations are somewhat simpler in composition, but are nevertheless also subject to problems of interfering activities.

Prentice *et al* (1980) have utilized a ß-glucanase complex from *Trichoderma reesei* (formerly *T. viride*) in the determination of ß-glucan from barley. This complex degrades extracted ß-glucan to glucose; the glucose is then determined by a chromatographic method. The method as outlined requires a preliminary extraction in carbonate buffer for 20 hours at pH 10. The resulting extract is dialyzed for 48 hours before precipitation of

the polymer, which then must be redissolved in the appropriate buffer before enzymatic hydrolysis for 10 hours. The lengthy extraction and dialysis steps required, coupled with the potential for alkaline degradation of the ß-glucan, make this procedure unattractive for routine analysis of ß-glucan.

The method developed by Martin and Bamforth (1981) also involves lengthy extraction and dialysis steps. A 40 hour extraction in 1.5% hydrazine at 104°C, while undoubtedly solubilizing all of the polysaccharides in the barley flour, is also likely to be inflicting substantial damage on the polysaccharides in question. Palmer and MacKenzie (1986) reported losses of 12% of ß-glucan in a hydrazine extraction and dialysis under much gentler conditions. After twenty-two hours of dialysis, cellulase from *T. reesei* (heated at 70°C to inactivate amyloglucosidases) was added to the dialyzed extract, which degraded ß-glucan completely to glucose. Problems were encountered with adsorption of glucose to the cellulase, which resulted in less than theoretical recoveries of glucose. These problems were overcome by the use of standardized enzyme concentrations and incubation times, and the generation of a new calibration curve for each batch of enzyme.

The procedure of Ahluwalia and Ellis (1984) offered a substantial improvement over previous extraction and digestion methods. They reported that all of the ß-glucan could be extracted in 50 mM perchloric acid at 96°C in 3 minutes, and that extraction for 10 minutes did not increase the amount of ß-glucan extracted. This contradicts the work of Jorgensen and Aastrup (1988b), who reported that the amount of ß-glucan extracted increased with increasing extraction time up to 10-12 minutes, whereupon the apparent ß-glucan content of the extract began to decrease with time, due to acid degradation of the polymer. The cellulase preparation used by Ahluwalia and Ellis (1984) was from *Penicillium funiculosum*, which degraded ß-glucan directly to glucose.

Anderson *et al* (1978) were among the first to use an enzymatic method to measure ß-glucan directly in barley, using a ß-glucan endohydrolase purified from *Bacillus subtilis*, and reported to be free of amylase and other polysaccharide degrading enzymes. The lichenase used was very specific for mixed-linkage ß-glucan, and depolymerized the ß-glucan to soluble oligosaccharides, which were then extracted and hydrolyzed to glucose. In this method, samples were initially extracted for 7 hours in 80% ethanol and dried; aliquots were hydrated and autoclaved for 1 hour to render the ß-glucan more accessible to the enzyme. Each sample was then incubated for 24 hours with ß-glucan endohydrolase, and the oligosaccharides generated were extracted in ethanol, dried, and hydrolyzed for 4 hours in 1N sulfuric acid. The resulting glucose was quantitated enzymatically using glucose oxidase and peroxidase. Although this method was highly specific for ß-glucan, the length of the total procedure precluded its use for routine determinations.

Because of the commercial unavailability of pure enzyme preparations at the time, Henry (1984) purified a ß-glucanase from bacterial amylase to assay ß-glucan directly in barley flour. Endogenous enzymes in the flour were first inactivated by treating the sample with boiling 80% ethanol for 5 minutes. This step also served to remove soluble sugars that could lead to high blank values in the assay. Purified enzyme was then used to degrade ß-glucan in barley flour (in a one-hour incubation) completely to oligosaccharides which were then estimated as reducing sugars by reaction with *p*-hydroxybenzoic acid hydrazide. No increase in reducing sugars was observed with longer incubation times (up to 4 hours). This method was later modified by the addition of a pre-reduction step using alkaline sodium borohydride, which had the effect of removing interference from reducing sugars without the need for an actual extraction

(Henry and Blakeney 1986; Henry and Blakeney 1988). The initial removal of reducing sugars was found to be particularly important in the assay of ß-glucan in malt, which contains a high proportion of degraded starch and ß-glucan. The sodium borohydride reduction step also inactivated endogenous enzymes in the sample. This method is based on the assumption that the ß-glucan from all varieties of barley would give similar or identical oligosaccharide profiles after lichenase digestion. However, recent reports suggest that the concentration of individual oligosaccharides from total ß-glucan varies between barley and oat ß-glucan and lichenan, and also between different barley varieties (Edney *et al* 1991, Wood *et al* 1991a).

An assay proposed by McCleary and Glennie-Holmes (1985) uses consecutive enzymatic hydrolyses to degrade ß-glucans in barley directly to oligosaccharides and then to glucose, which can be determined enzymatically. A one-hour incubation with lichenase (from *B. subtilis*) depolymerises the ß-glucan in the sample to soluble oligosaccharides, which are removed from the digested sample by centrifugation. An aliquot of the supernatant is then incubated with ß-glucosidase for twenty minutes to hydrolyse the ß-oligoglucosides to glucose; the ensuing glucose is then determined using glucose oxidase and peroxidase. A preliminary, five minute boiling step is included in this method to inactivate endogenous enzymes which could lead to inaccurate results. Henry and Blakeney (1988) claimed that methods involving conversion of oligosaccharides to glucose and calibration against glucose probably underestimate ß-glucan in samples. This claim is based on the assumption that such methods result in the production of oligosaccharides with a d.p. greater than 10 which precipitate during enzyme hydrolysis (Woodward *et al* 1983b), and are thus excluded from the soluble oligosaccharide fraction. The addition of cellulase to depolymerize higher d.p.

oligosaccharides, however, did not increase the estimated ß-glucan content in the method of McCleary and Glennie-Holmes (1985). The initial removal of soluble sugars from the sample was also found to be unnecessary, as the soluble sugar levels found in flour made from unmodified grain were quite low. A commercial ß-glucan assay kit containing purified lichenase and ß-glucosidase (certified devoid of activity on starch, maltosaccharides, cellulose and sucrose: McCleary *et al* 1988) is now marketed by BioCon.

A group of Swedish researchers (Åman and Hesselman 1985; Åman and Graham 1987b) has developed and subsequently improved an enzymatic method of ß-glucan analysis using a commercial ß-glucanase. These methods overcame problems with impurities in commercial enzyme preparations by prior removal of starch through digestion at high temperatures with a heat stable α-amylase preparation (Termamyl). ß-Glucan degrading enzymes, known to be present in this α-amylase preparation, are inactivated at the temperature of incubation (boiling water bath). A four-hour incubation with amyloglucosidase to ensure complete starch degradation follows the Termamyl digestion, after which soluble polymers are precipitated overnight in ethanol. Complete digestion of the ß-glucan in the pellet (which includes the de-starched flour as well as the precipitated polymers) to glucose was then accomplished with one enzyme preparation. This method was reported to show high correlations with FIA for both oats (r=.94) and barley (r=.96) (Åman and Graham 1987b). Nevertheless, even the improved protocol of these workers was over-long for routine determinations.

2.2.5 Indirect methods for determination of ß-glucan

Indirect measurements of a particular component require, at least initially, a direct method to be used for calibration. Alternatively, they measure a constant proportion of the component in question, or a second component which has been shown to correlate well with the component of interest. The majority of indirect methods for measurement of ß-glucan involve extraction of the polymer and measurement on the resulting extract. These methods suffer in that no method currently exists for quantitative extraction of native ß-glucan that is uncontaminated by starch (Wood 1986).

Bendelow (1975) extracted ß-glucan from barley flour with a mixture of α-amylase (heat treated to inactivate ß-glucanases) and papain at 50°C, followed by addition of TCA to 7.5% to precipitate proteins. The "extractable" ß-glucan in the resulting solution was measured viscometrically. Although the α-amylase used in this method was heat treated to inactivate ß-glucanases, no mention was made of the possibility of degradation of ß-glucans by endogenous ß-glucanases, which could have a significant effect on the viscosity of the final extract. The possible contribution of other soluble components in the extract to viscosity was similarly disregarded.

Morgan and Gothard (1977) extracted ß-glucan from ground barley in HCl-KCl buffer, pH 1.5 at 40°C for 4 hrs. The extract was allowed to equilibrate to room temperature and settle. The supernatant was drawn into a tube and the time required for a falling ball to move a certain distance in the tube was measured. The relationship between falling time and viscosity as measured by a Brookfield viscometer was logarithmic. Since ß-glucan content was also related to the logarithm of the viscosity, it was concluded that there was a direct relationship between falling time and ß-glucan content. It seems likely that the use of one indirect method to calibrate a second indirect

method increased the level of error in a system already subject to inaccuracy.

Aastrup (1979) found that the viscosity of an acid flour extract at room temperature was highly correlated to the soluble ß-glucan content, but not the insoluble ß-glucan content of barley. Total and soluble ß-glucan were also measured by the enzymatic method of Anderson *et al* (1978), and the insoluble ß-glucan calculated from the difference. The correlation between the log of the extract viscosity and soluble ß-glucan yielded an r^2 of 0.99. According to Aastrup (1979), this relationship implied that as ß-glucan increased, the sensitivity of the measured viscosity to differences increased as well. The relationship between viscosity and total ß-glucan was not as strong ($r^2 = 0.78$), and no correlation was observed between extract viscosity and insoluble ß-glucan.

A problem inherent in all of the viscometric methods discussed is that only the soluble ß-glucan in a sample is measured. The proportion of the total ß-glucan which is soluble tends to be variable between different types of grains, or even different cultivars of the same grain (Anderson *et al* 1978). In addition, both the amount of ß-glucan extracted, and also its molecular structure are dependent upon extraction conditions (Aastrup 1979), which vary widely among methods; viscometric values vary with the molecular weight of the ß-glucan (Madacsi *et al* 1983). The possibility of co-extraction of other components which may contribute to the viscosity of an extract is generally disregarded in most cases, often without adequate supporting data. In general, because of the variable relationship between ß-glucan concentration and viscosity, these methods cannot be used to provide reliable estimates of ß-glucan content (Wood 1986).

One of the simplest indirect methods proposed to date is near-infrared analysis using the commercially available InfraAlyzer which is marketed to estimate protein, moisture, oil and fibre in various products. Allison *et al* (1978) proposed a rapid means

of estimating soluble ß-glucan in ground samples by relative measurements of near-infrared reflectance at six different wavelengths. Values were compared with viscosity which, when plotted on a semi-logarithmic scale, yielded a linear calibration curve. This method is rapid and simple: once the machine has been calibrated, no chemical analysis is necessary. The method as published however, had a number of weaknesses. While the reported correlations between observed and predicted values for extract viscosity and ß-glucan were high (r=.81 and r=.85 respectively) and indeed could be considered useful for preliminary screening in some cases, the method is insufficient where more accurate measurements of ß-glucan content are required. The authors did not derive a calibration curve from their own data, but used a previously published equation (Greenberg and Whitmore 1974) to relate viscosity to soluble ß-glucan content. Most importantly, the method did not measure total ß-glucan, but only the soluble component. This, as noted above, can vary considerably depending on the conditions of extraction (see Aastrup 1979).

2.3 Arabinoxylans in Cereals

2.3.1 Occurrence

In addition to ß-glucans, arabinoxylans are important cell wall matrix polysaccharides in the grasses. The arabinoxylans and glucuronoarabinoxylans are frequently referred to as pentosans because their main constituents are the pentoses arabinose and xylose. In some cases, however, galactose and glucuronic acid or its 4-O-methyl- ether are also present, although in smaller amounts (Aspinall 1970; Fincher and Stone 1986). Arabinoxylans represent the major hemicellulosic polysaccharide in non-endospermic cell walls of the grasses (Aspinall 1970; Dey and Brinson 1984; Wilkie

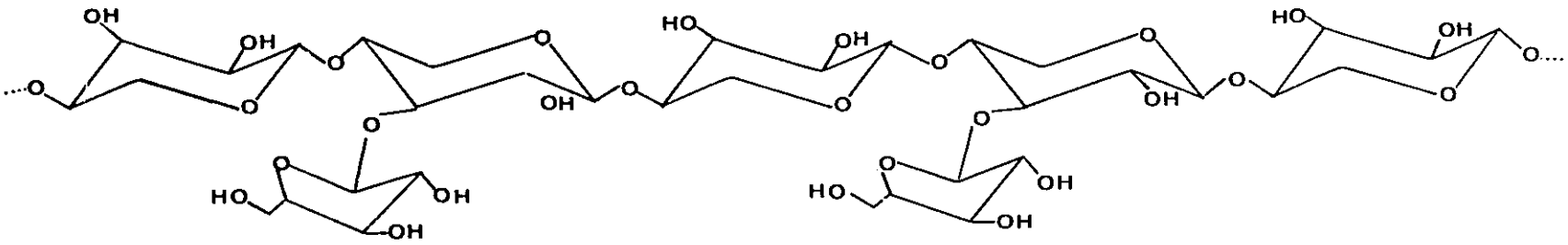
1985b). They are also found in bran and endosperm tissues. Arabinoxylans have been reported in seed tissues of wheat, barley, rice, rye and oats (Perlin 1951a,b; Mares and Stone 1973a; Fincher 1975; D'Appolonia and MacArthur 1975; Kim and D'Appolonia 1976; Ballance and Manners 1978; Shibuya and Misaki 1978; Bacic and Stone 1980; MacArthur and D'Appolonia 1980; Selvendran and DuPont 1980; Bacic and Stone 1981a; Brioullet and Mercier 1981; Brioullet *et al* 1982; Shibuya *et al* 1983; Aspinall and Carpenter 1984; Ahluwalia and Ellis 1985; Ahluwalia and Fry 1986; Henry 1987; Delcour *et al* 1989). A comparative study of concentrations of pentosan in endosperm and whole grain of wheat, barley, oats and rye showed that the highest levels in both whole grain and endosperm were found in rye, and the lowest levels were found in oats (Henry 1987). Whole kernels of oats contained 5.8% pentosan, while the endosperm content was very low, at 0.7%. The low pentosan content of oats was previously noted by other workers (Preece and MacKenzie 1952b; Preece and Hobkirk 1953; MacArthur and D'Appolonia 1980).

2.3.2 Physicochemical Properties

Molecular weights ranging from 20,000 to 170,000 have been reported for cereal arabinoxylans, depending on the fraction isolated, and the source (reviewed by Fincher and Stone 1986). In general, arabinoxylans in the grain are of lower molecular weight than the ß-glucans.

A linear chain of ß-(1→4)-linked xylopyranosyl units (Fig. 3) makes up the backbone of the molecule, and α-L-arabinofuranosyl and other residues are attached through O-2 and O-3 atoms of the xylosyl residues (Medcalfe and Gilles 1968; Aspinall 1970; Wilkie 1935a; Fincher and Stone 1986). Single arabinosyl units are thought to be mostly linked to the O-3 atoms of the main xylan chain, but a significant proportion of the

Figure 3. Diagram illustrating the chemical structure of cereal arabinoxylans.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xylosyl residues are doubly substituted at atoms O-2 and O-3 (Wilkie 1985b; Fincher and Stone 1986). Glucuronic acid and its 4-O-methyl- ether, which together are usually less than 2% of the total arabinoxylan, are linked to O-2 atoms of the xylosyl residues (Aspinall 1970; Fincher and Stone 1986). Galactose, when present, is found as the terminal residue in disaccharide side chains of D-galactosyl-L-arabinofuranose, and D-xylopyranosyl-L-arabinofuranosyl side chains are also reported (Aspinall 1970; Fincher and Stone 1986). Ferulic acid is found associated with isolated arabinoxylan, and is esterified to arabinosyl residues on the xylan backbone (Geissmann and Neukom 1973a; Ciacco and D'Appolonia 1982; Ahluwalia and Fry 1986). Esterified ferulic acid has been implicated in oxidative gelation of cereal pentosans (Geissmann and Neukom 1973b; Hoseney and Faubion 1981; Ciacco and D'Appolonia 1982).

Arabinoxylans form highly viscous aqueous solutions (Medcalfe *et al* 1968), a property which is attributed to their conformational asymmetry, since most cereal arabinoxylans carry little or no charge (Fincher and Stone 1986). A water-soluble arabinoxylan from wheat flour was shown to exist in solution as a fully extended rod (Andrewartha *et al* 1979). Unsubstituted xylan is highly insoluble, and forms a three-fold, left-handed helix in the solid state (Marchessault and Settineri 1964; Settineri and Marchessault 1965). Arabinosyl, and presumably, to a lesser extent, other substituents, appear to force the xylan into a more extended conformation, and increase the solubility of the molecule as well (Perlin 1951b; Neukom *et al* 1967; Dea *et al* 1973; Andrewartha *et al* 1979).

3. STUDIES ON ß-GLUCAN IN DOMESTIC AND PRIMITIVE OATS

3.1 Introduction

ß-Glucan is the major component of the soluble dietary fibre in oats (Wood 1986). With the current interest in the reported clinical benefits to be derived from including oat fibre in the diet (Kirby *et al* 1981; Anderson and Chen 1986, Anderson and Gustafson 1988; Gold and Davidson 1988; Wood *et al* 1989, Anderson *et al* 1990; Demark-Wahnefried *et al* 1990; Wood *et al* 1990; Davidson *et al* 1991; Van Horn *et al* 1991) comes an increased requirement for more rapid and accurate methods to assay ß-glucan in oats, and also for more information on the range of ß-glucan contents available in oats.

Because of the importance of barley ß-glucan in the brewing industry, most of the methods reported in the literature for the measurement of ß-glucan in cereals were developed for barley. Differences between the ß-glucans of different cereals have been reported, however, particularly with respect to solubility (Wood *et al* 1977; Anderson *et al* 1978; Åman and Graham 1987a; Wood *et al* 1991a). It was therefore necessary to ensure that the assay used for the experiments in this thesis provided an accurate estimation of the total ß-glucan in oats. Of the assays reported in the literature, some appeared to provide a very good estimate of total ß-glucan, but suffered the disadvantage of lengthy and tedious procedures (Anderson *et al* 1978; Åman and Graham 1987b). The enzymatic assay of McCleary and Glennie-Holmes (1985), which, although more rapid than other assays, is still somewhat time-consuming, was ultimately considered the logical starting point for the experiments proposed in this thesis. This assay has the added advantage of commercial availability in a kit containing enzymes of certified purity.

In the present study, the specificity of the enzymatic assay was confirmed by testing a selection of different α- and ß-linked glucans. The assay was then modified to estimate the maximum amount of ß-glucan (presumed to be the total) by adjusting the time of lichenase digestion, and the grinding procedure. The assay was then used to measure the ß-glucan content in a number of oat cultivars, grown in 5 locations over 3 years, to assess the genetic and environmental variability of oat ß-glucan. Further possible sources of genetic variability were investigated in 18 primitive species of *Avena*, grown in the same location in the same year to eliminate environmental influences. A second method using Flow Injection Analysis (FIA), which is simpler and less time consuming than the enzymatic method, has been reported to give results in good agreement with those from enzymatic assays of ß-glucan in both oats and barley (Åman and Graham 1987; Jorgensen 1988; Jorgensen and Aastrup 1988a; Sendra *et al* 1989; Switala *et al* 1989; Anderson 1990). FIA was used to measure ß-glucan content in many of the same samples that were assayed enzymatically. When the results were compared, it was concluded that while FIA was a useful method for screening relative amounts of ß-glucan in samples, the enzymatic assay provided a more accurate estimation of the total ß-glucan in oats.

In addition to genetic and environmental variation, structural variation in oat ß-glucans was assessed in both domestic and primitive oats by comparing the molar ratios of trisaccharides and tetrasaccharides released during lichenase digestion. Tri- and tetrasaccharides represent 90% of the oligosaccharides released by the action of lichenase on ß-glucan from either oats (Wood *et al* 1991a) or barley (Woodward *et al* 1983b), and thus constitute the major structural repeating units of the polysaccharide.

Differences in distribution of ß-glucan in kernels of oats, barley and wheat were examined using microspectrofluorometric analysis of wall-bound Calcofluor. The relative fluorescence intensity (RFI) of bound Calcofluor has been reported to be approximately proportional to the amount of ß-glucan present (Wood and Fulcher 1978; Jensen and Aastrup 1981; Wood and Weisz 1984; Wood 1985; Jorgensen 1988).

The results of studies reported in this section provide an improved understanding of oat ß-glucan, and have potential applications from the standpoints of both production and utilization of oats and oat products.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Plant Materials

Domestic Oats: Samples of six registered domestic Canadian oats (*Avena sativa* L.) as well as 7 unregistered lines grown at 5 locations over 3 years were obtained from the Eastern Cooperative Oat Test (Agriculture Canada). The cultivars used were the registered varieties Donald, Marion, Ogle, Shaw, Tibor, Woodstock, and the unregistered lines OA447-27, OA516-2, QO191.70, QO199.27, QO199.60, QO220.9 and QO220.13. The oats were grown at locations in Ontario (Ottawa, Kapuskasing, Ailsa Craig), Quebec (Ste. Rosalie) and Prince Edward Island (Charlottetown), in 1984, 1985 and 1986. The variations in location and growing season provided a range of environmental conditions during growth. Samples (approx. 2 kg) were obtained from each location, and were subsequently divided into smaller samples prior to dehulling and assay. A sample divider (Humboldt Testing Equipment, Chicago, Ill.) was used to obtain random, representative samples of all cultivars.

Primitive Oats: Accessions of primitive oats were obtained from Plant Gene Resources, Agriculture Canada. Eighteen species of *Avena* including 9 diploids (*A. canariensis, A. clauda, A. eriantha, A. hirtula, A. longiglumis, A. lusitanica, A. pilosa, A. strigosa, A. ventricosa*), 4 tetraploids (*A. barbata, A. magna, A. murphyi, A. vaviloviana*) and 5 hexaploids (*A. byzantina, A. fatua, A. hybrida, A. occidentalis, A. sterilis*) were germinated on moist filter paper then transplanted to pots when roots started to show. When the seedlings were approximately 2-5 cm high, the pots were moved into a growth chamber for vernalization (16 hrs light, 2°C; 8 hrs dark, 0°C) for 4 weeks before transplanting in the field, to optimize growth and heading (Sampson and Burrows 1972). The plants were grown in random plots in a field at the Central Experimental Farm, Agriculture Canada, Ottawa, in the 1988 growing season, and seeds were harvested as they matured to prevent loss and cross-contamination due to shattering.

3.2.2 Enzymatic analysis of ß-Glucan

The ß-glucan content of the oat samples was determined using a modification of the lichenase digestion method of McCleary and Glennie-Holmes (1985). Samples were dehulled manually; the resulting groats were ground twice to pass a 1mm screen in a UDY Cyclone grinder (UD Corporation, Colorado). The groat flour was dried in a vacuum oven at 70°C for 4 hours prior to analysis. Enzymes and standards from the Biocon ß-Glucan Assay Kit (Biocon, U.S.) were used. Aliquots (100 mg) were weighed into tubes, and 200 µL of 50% ethanol was added to wet the flour. Aliquots of 2.0 mL of sodium phosphate buffer (20 mM, pH 6.5) were added, as well as small magnetic stirring vanes, to each tube, and the tubes were placed in a boiling water bath and stirred for 5 minutes. After cooling, 50 µL of lichenase (50 units/mL) was added, and the tubes incubated with

stirring at 40°C for 2.5 hours. At the end of the incubation period, the volume was adjusted to 10 mL with 0.2% (w/v) benzoic acid, and the tubes vortexed vigorously. The samples were centrifuged at 10,000 x g for 5 minutes, and the pellet discarded.

For automated determinations, a Technicon System II Autoanalyzer (Technicon Corp, Tarrytown, N.Y.) was used. A 1 mL aliquot of the supernatant was incubated with 1 mL of sodium acetate buffer (50 mM, pH 4.5) and 50 µL of ß-glucosidase (2 units/mL) at 40°C for 20 minutes. At the end of the incubation, these samples were placed directly on the Autoanalyzer, and the glucose determined using a glucose oxidase/peroxidase system with 4-aminophenazone as the colour reagent. Reagents were as follows:

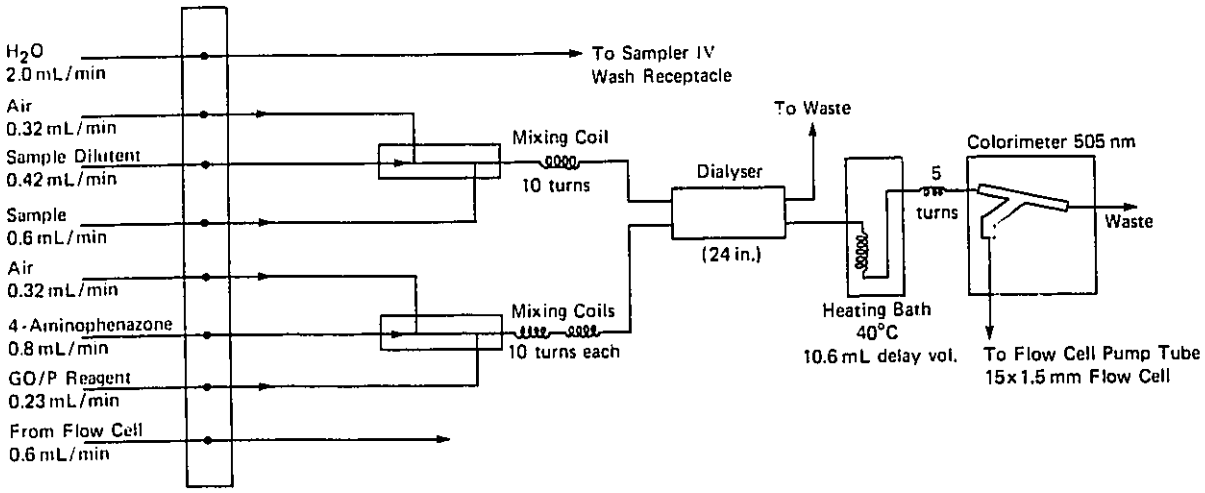
a) Glucose oxidase/peroxidase reagent; 5.0 mg of glucose oxidase (Grade I, Boehringer-Mannheim, Dorval, Québec), and 1.0 mg of horseradish peroxidase (Boehringer-Mannheim) were dissolved in 50 mL of pH 7.0 buffer (20.64 g of disodium hydrogen phosphate heptahydrate, 10.97 g of sodium dihydrogen phosphate monohydrate, 4.0 g of benzoic acid, and 3.0 g of parabenzoic acid in 2 L of water).

b) 4-aminophenazone; 0.1 g/L of 4-aminophenazone (Sigma Chemical Co.) was dissolved in water containing 1mL/L Brij 35 (Fisher).

A schematic diagram of the automated system is presented in Fig. 4.

For manual determinations, the ß-glucosidase digestion and glucose determination were performed as outlined in the Biocon instruction pamphlet. Aliquots of 100µL of supernatant and 100µL of sodium acetate buffer were incubated with 50µL of ß-glucosidase for 20 minutes at 40°C. Aliquots of 3mL of glucose oxidase/peroxidase reagent (glucose oxidase, 6250 U/L; peroxidase 1250 U/L; 4-aminophenazone, 0.1 g/L; buffer as for automated assay) were added and, after incubating for 20-30 minutes (optimum time was determined for each new batch of enzyme), the absorbance was read at 510 nm.

Figure 4. Flow diagram for the automated analysis of glucose using the glucose oxidase/peroxidase assay. (From Wood et al 1991a).


H_2O
2.0 mL/min
Air
0.32 mL/min
Sample Diluent
0.42 mL/min
Sample
0.6 mL/min
Air
0.32 mL/min
4-Aminophenazone
0.8 mL/min
GO/P Reagent
0.23 mL/min
From Flow Cell
0.6 mL/min
To Sampler IV
Wash Receptacle
Mixing Coil
10 turns
Mixing Coils
10 turns each
Dialyser
(24 in.)
To Waste
Heating Bath
40°C
10.6 mL delay vol.
5
turns
Colorimeter 505 nm
Waste
To Flow Cell Pump Tube
15x1.5 mm Flow Cell

All enzymatic analyses were performed in triplicate. A typical regression equation for a glucose standard curve is as follows:

$$ABS_{510} = 0.862 \times \text{glucose}(\mu g/\mu L) - 0.0034 \quad (r=0.9999)$$

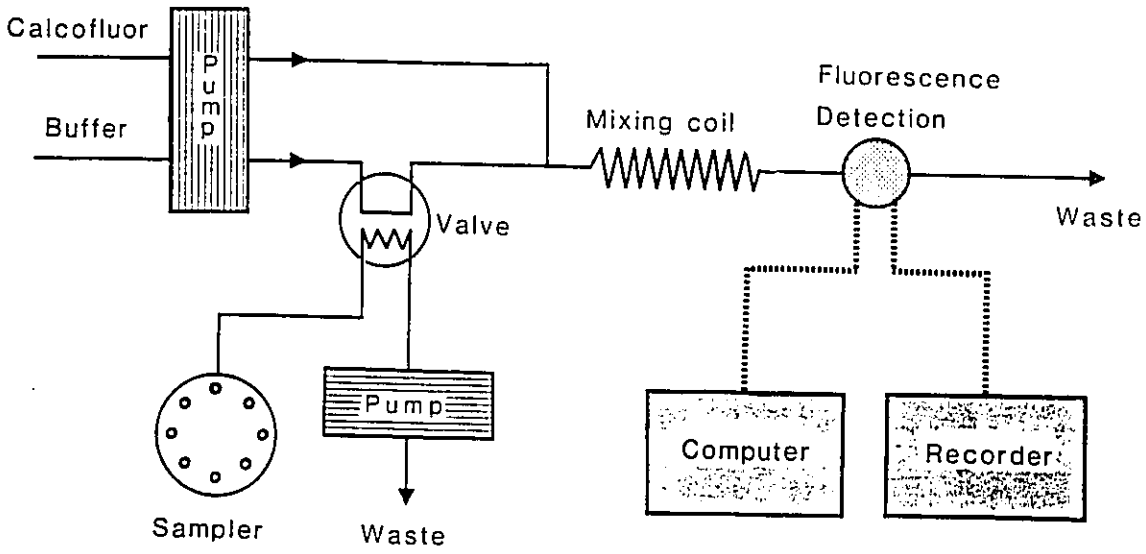
3.2.3 Flow injection analysis of ß-glucan

In the determination of ß-glucan content using flow injection analysis (FIA), groat flour samples were prepared and dried as for the enzymatic method described above. Aliquots of 100 mg of flour were weighed into Pyrex screw cap test tubes, and 9.9 mL of distilled water plus 100 µL of heat stable α-amylase (Termamyl, Novo Industri a/s, Copenhagen, Denmark) was added, and the screw cap tightly mounted. The samples were mixed thoroughly using a vortex mixer, and incubated in a boiling water bath for 1 hour. At the end of the incubation period, the samples were cooled to room temperature, and 10 mL of 75 mM sulfuric acid was added. The tubes were again tightly sealed, and mixed thoroughly before incubating for a further 10 minutes in boiling water. The tubes were cooled rapidly to room temperature and the contents mixed thoroughly using a vortex mixer. Aliquots of 10 mL were removed and centrifuged for 10 minutes at 3000 x g. The supernatant from these samples was introduced directly into the ß-Glucan Analyzer (Carlsberg, Copenhagen, Denmark), a dedicated FIA system, for analysis. A schematic diagram of the Carlsberg FIA system is presented in Fig. 5. FIA analyses were performed in duplicate.

3.2.4. Determination of tri:tetrasaccharide ratios

Dried groat flour (prepared as above) was refluxed in 80% ethanol in a boiling water bath for two 1 hour periods. The flour was washed with 80% ethanol and

Figure 5. Flow diagram for the analysis of ß-glucan by flow injection analysis using the Carlsberg ß-Glucan Analyzer. (From Jorgensen 1988).


Calcofluor
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Pump
Mixing coil
Fluorescence
Detection
Waste
Valve
Pump
Sampler
Waste
Computer
Recorder

centrifuged (10,000 rpm, 5 mins), then 2.0 mL of sodium phosphate buffer and 100 µL of lichenase were added to the pellet. The samples were incubated at 40°C with stirring for 3 hours. The volume was adjusted to 10 mL with distilled water, and the samples vortexed vigorously, then centrifuged (10,000 rpm, 5 min) and the pellet discarded. The supernatant was filtered through 0.45 µm filters before HPLC analysis.

Anion exchange chromatography was performed with a Dionex GPM-2 Gradient Pump and a Dionex CarboPac PA1 column (4 x 250 mm). The eluant was 150 mM NaOH at a flow rate of 1.0 mL/min. Injections were made using a Dionex autosampler, and oligosaccharides were detected using a Dionex PAD-2 Pulsed Amperometric Detector (Gold). A typical oligosaccharide separation is shown in Fig. 6.

Information on protein (Kjeltec) and oil (Soxhtec) contents of domestic oats was obtained from the Eastern Co-operative Oat Test.

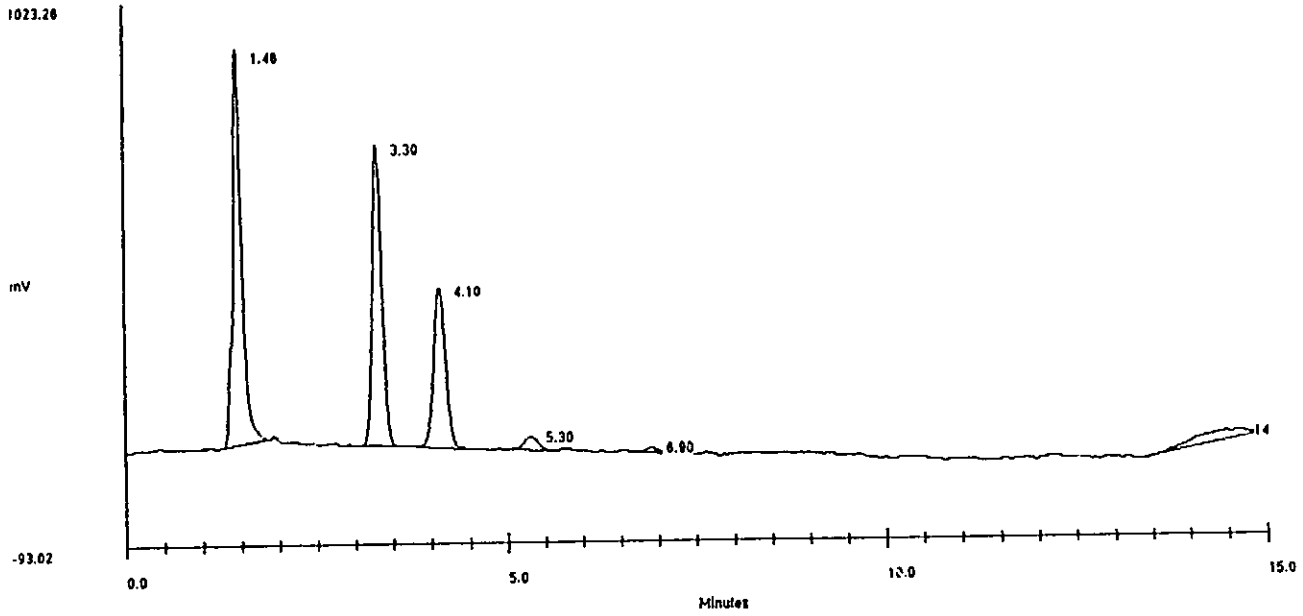
Thousand kernel weights were calculated by weighing 150 kernels of each sample. **Protein determinations** for primitive oats (dehulled) and selected domestic samples were performed in duplicate using a Kjeltec Auto 1030 Analyzer (Tecator, Sweden).

3.2.5 Distribution of ß-glucan within the caryopsis

Oat samples of known ß-glucan content (cultivars: Donald, 3.68%; OA516-2, 3.98%; Tibor, 4.56%; Woodstock, 5.07%; and Marion, 6.35%) were obtained from the Eastern Cooperative Oat Test (Agriculture Canada) and embedded in blocks of polyester resin. Barley samples were the generous gift of Dr. S. Aastrup, Carlsberg Research Laboratory, Copenhagen, Denmark (cultivars: M-737, 2.8% ß-glucan; Chalky Glen, 3.2%; Minerva, 6.0 %; and Arizona Hull-less, 11.0%.), and from Agriculture Canada (Leger,

Figure 6. Chromatogram showing the separation of gluco-oligosaccharides by HPLC using PAD.

PEAK NUM	RET TIME	PEAK NAME	CONC. in ug/ml	AREA	HEIGHT	BL	REF PEAK	% DELTA RET TIME
2	3.30	trisaccharide	5.289e+001	5.428e+006	612777	1	2	0.00%
3	4.10	tetrasaccharide	5.438e+001	3.508e+006	321912	1	2	0.00%



5.7%). Wheat samples were obtained from Agriculture Canada. Only kernels with no visible fungal contamination were chosen for analysis.

To position seeds for embedding, plasticene was rolled flat, and a shallow cardboard box (sides approximately 4 cm long, 1.5 cm deep, open at both ends) was pushed into the surface such that a tight seal was achieved between the cardboard and the plasticene. Kernels were held vertically and the embryo end was pushed lightly into the plasticene.

Polyester resin was mixed with colour (white) and hardener according to the manufacturer's directions (Motomaster Liquid Auto Body Resin, Canadian Tire Corporation) and poured into the box completely covering the oats. After the resin had hardened, the cardboard was removed, and the plasticene was carefully scraped off (so as not to damage the embryos). Heavy tape was used to build a shallow wall around the exposed end of the block, and the resulting area filled with more resin so that the kernels were completely enclosed in resin.

For microspectrofluorometry, the blocks were abraded to the required depth using a belt sander (Black and Decker, 60 x 400 mm), and the exposed end polished by hand with fine sandpaper. Through-out the sanding procedure, a level was used to keep the depth of the block as consistent as possible. The exposed surface of the blocks were treated for 2 minutes with .01% Calcofluor in 50% phosphate (50 mM, pH 8) buffered ethanol, then rinsed with 50% ethanol. The blocks were then counterstained for 30 seconds with Fast Green FCF (0.1% in 50 mM acetate buffer, pH 4.0), rinsed with distilled water and the exposed surfaces blotted dry. The blocks were placed on the scanning stage of a Zeiss UMSP80 scanning microspectro-photometer equipped with an epi-illuminating condensor, HBO 100-W mercury illuminator and controlled by a Hewlett-

Packard mini-computer. A filter combination with excitation wavelength 365 nm and emission ≥420 nm was used. A 10X Neofluar objective was used for scanning. The microspectrophotometer was calibrated at the beginning of each session using a solution of .002% 4-methylumbelliferone (Sigma) (freshly diluted from a stock solution of 0.01%, made up weekly). A 2500x3000 μm field was marked over each kernel cross-section, and scanned in a matrix fashion to generate a plot of fluorescence intensities across the field.

3.2.6 Preparation and examination of methacrylate-embedded samples

For microscopic examination of seed sections, samples were fixed and embedded in glycol methacrylate (GMA) as described by Fulcher and Wong (1980). Briefly: pieces of oat kernel (no more than 2mm thick) were fixed in 6% glutaraldehyde in sodium phosphate buffer (25 mM, pH 7.0) at 4°C for 72 hours. Fixed samples were dehydrated sequentially in methyl cellosolve, ethanol, *n*-propanol and *n*-butanol (24 hours each), infiltrated with GMA monomer (Feder and O'Brien 1968) for 72 hours, and polymerized at 60°C overnight after exclusion of air from the surface of the samples. Sections 3 μm thick were cut using glass knives, and mounted on slides.

Sections were examined using a Zeiss Universal Research Microscope (Carl Zeiss Canada Ltd.). The microscope was equipped with a tungsten illuminator for examination of specimens by bright-field microscopy using transmitted light, and a IIIRS epi-illuminating condenser for fluorescence analysis. The fluorescence filter combination used for examination of autofluorescence and Calcofluor stained samples had a dichromatic beam splitter, and an exciter/barrier filter set with maximum transmission at 365 nm/ 420 nm. Photomicrographs were obtained using 35 mm Kodak Ektachrome 100 for bright-field images, and Ektachrome 400 for fluorescence micrographs.

3.3 Results and Discussion

3.3.1 Development of ß-glucan assay procedures for genetic and environmental screening

3.3.1.1. Optimisation of enzymatic assay method

Specificity of lichenase digestion: The specificity of the enzymatic assay for the measurement of ß-glucan is conferred by the (1→3),(1→4)-ß-D-glucan 4-glucanohydrolase (lichenase) used in the first digestion step of the assay. Because the ß-glucan is assayed in flour from groats rather than on an extracted and isolated polysaccharide, the purity of the lichenase used is of prime importance. Contamination of the enzyme with cellulolytic or amylolytic activity could result in over-estimation of ß-glucan in the sample.

Table 1 shows the release of glucose from a variety of ß- and α-linked glucans after digestion with lichenase followed by ß-glucosidase, which cleaves oligosaccharides released by lichenase to glucose monomers. The only polysaccharides in which significant amounts of glucose were released by this treatment are barley ß-glucan and lichenan from Icelandic moss, both of which are mixed linkage (1→3),(1→4)-ß-D-glucans. The lichenase/ß-glucosidase combination marketed by Biocon is essentially free of troublesome contamination, as indicated by a glucose release of less than 0.5% of the non-ß-glucan samples (Table I). Of particular importance among the polysaccharides that did not yield glucose are cellulose and starch, both of which are present in the samples used for the assay of ß-glucan. The glucose values shown for barley ß-glucan and lichenan from Icelandic moss (853 µg/mg and 719 µg/mg sample respectively) suggest that these samples (obtained from Sigma) contained some impurities. No attempt was made to verify the presence, or ascertain the nature, of impurities in any of the

Table 1. Specificity of Biocon lichenase. Glucose released (μg/mg sample) by lichenase digestion (one hour) followed by ß-glucosidase digestion (20 mins) of different glucans, both linear (lin) and branched (br). Values represent the mean (n=3) ± standard deviation.

Polysaccharide	Linkage(s)	Glucose (μg/mg sample)
Cellulose	ß-(1-4) lin	0.2± 0.2
Dextran	α-(1-6),(1-3) br	1.3± 1.8
ß-Glucan(barley)	ß-(1-3),(1-4) lin	853.0±48.4
Laminarin	ß-(1-3) lin	0
Lichenan	ß-(1-3),(1-4) lin	718.7± 8.8
Pachyman	ß-(1-3) lin	0.8± 0.1
Pullulan	α-(1-4),(1-6) lin	2.7± 2.3
Starch(soluble)	α-(1-4),(1-6) br	0.7± 0.3

polysaccharides used. Pure samples of all of the polysaccharides listed in Table 1 should contain only glucose.

Conditions of lichenase digestion: Because availability of samples in some cases can be limited, the enzymatic assay procedure as specified in the Biocon kit was scaled down with respect to quantities. A second, but not inconsiderable, advantage to this scaling down was a sizeable increase in the number of assays which could be performed with each kit, and a resulting decrease in the overall expense per assay.

In addition to enzyme purity, the accurate estimation of ß-glucan content in a sample depends upon the accessibility of the substrate to the enzyme. In the oat samples assayed here, this accessibility could be limited by two obvious factors: incomplete dispersion of the enzyme because of solution viscosity, or incomplete penetration of the enzyme into large particles.

The first step of the enzymatic assay is an incubation of the sample with buffer in a boiling water bath for 5 minutes. This step serves a dual purpose: inactivation of endogenous enzymes in the sample, and solubilization of a portion of the ß-glucan in the sample so that both the soluble and insoluble ß-glucans are more accessible to the enzyme. Any starch in the sample is also gelatinised by this treatment. The solubilization of ß-glucan and gelatinisation of the starch in the sample result in increased sample viscosity which is particularly noticeable when the sample is cooled to 38°C for the enzyme digestion.

When samples of oat flour were compared with barley standards, it was consistently but not quantitatively observed that at the end of the heat inactivation step, the oat flour/buffer suspension was much thicker than the barley flour/buffer suspension.

Extracts from oats were previously reported to have a higher viscosity than extracts from barley (Wood *et al* 1978). The higher viscosity of the oat samples relative to barley is due, at least in part, to the higher proportion of soluble ß-glucan in oats (Åman and Graham 1987a). Concern that the high viscosity would impede the dispersion of enzyme through the sample and possibly lead to low or inconsistent ß-glucan values dictated an experiment to investigate the effect of length of lichenase digestion time on the determination of ß-glucan in oat samples. Table 2 shows the effect of lichenase digestion time on the final ß-glucan value obtained for two cultivars of oats with different levels of ß-glucan. In general, the ß-glucan value obtained increased with increasing digestion time from an initial time of 0.5 hrs, and levelled off after 2.5 hrs. While the absolute differences in ß-glucan content were not found to be statistically significant, the trend was remarkably consistent in a number of trials. The consistent increase in the estimated ß-glucan content of the oat samples which accompanied the increase in lichenase digestion time suggested that sample viscosity may impede enzyme dispersion to some extent, and that incubation times longer than the 1.0 h recommended by Biocon were necessary to ensure accurate and consistent estimates of ß-glucan content in oat samples. All subsequent lichenase digestions were, therefore, incubated for 2.5 hours.

Conditions of grinding: A second concern regarding enzyme penetration was the size of the particles in the ground groats: smaller particle sizes might allow the enzyme easier access to the ß-glucan in the sample. To explore this possibility, samples of oats were ground to pass screens of decreasing mesh size. Because of the relatively high lipid content of the oats, problems were encountered with clogging of the screens as the

Table 2. Effect of lichenase digestion time on yield of ß-glucan. Samples were digested with lichenase for increasing periods of time, followed by ß-glucosidase digestion and determination of glucose. ß-Glucan values were calculated from glucose. Values represent the mean ± standard deviation. (n=3, except for Marion at 3.5 hrs, which represents a single determination).

Digestion Time (hrs)	% ß-glucan (dry wt. basis)	
	Marion	Donald
0.5	3.7±.84	3.2±.05
1.0*	4.8±.09	3.4±.01
1.5	4.9±.07	3.4±.09
2.0	5.0±.08	3.4±.01
2.5	4.9±.15	3.4±.09
3.0	4.8±.12	3.5±.13
3.5	4.8	3.5±.03
4.0	4.9±.05	3.6±.02

* time recommended in Biocon assay kit

mesh size decreased. To alleviate these problems, samples were ground to pass the largest mesh (1.1 mm) screen, and mixed with crushed dry ice before regrinding to pass the smaller mesh screens. This procedure also prevented damage to the finer screens caused by the impact of the kernels when they were first introduced to the grinder.

The effect of grinding and screen mesh size on yield of ß-glucan is shown in Table 3. Contrary to expectations, as the mesh size decreased, the yield of ß-glucan decreased also. This result indicated the selective retention of bran and cell wall material by the smaller screens. Indeed, this material could be seen on visual inspection of the inside of the grinder. It appeared that the abrasive head of the grinder was not sufficient to break the cell walls into small enough fragments to pass through the smaller mesh screens.

Attempts to use other types of grinders were unsatisfactory. When a Wiley mill (where sharp blades cut the sample against the screen) was used, a portion of the sample remained too large to pass through the screen in virtually all cases, regardless of screen mesh size or the time allowed to complete grinding. A coffee grinder enabled complete recovery of the sample, but did not grind the samples to a fine, consistent flour, and thus did not address the problem of particle size.

Inspection of the data from the UDY grinder (Table 3) suggests that particle size does have an impact on ß-glucan yield. The ß-glucan yield was higher when the sample was ground twice to pass the largest (1.1 mm mesh) screen (sample 1-1), than when the sample was ground only once (sample 1-0). Grinding twice to pass a relatively large mesh screen achieved the desired reduction in particle size without the selective loss of certain fractions which was observed with the smaller mesh screens. This grinding protocol was therefore followed in all subsequent ß-glucan assays (both enzymatic and Flow Injection Analysis).

Table 3. Effect of grinding on yield of ß-glucan. All samples were ground first to pass a 1.1 mm mesh screen. The flour was then reground to pass successively smaller mesh screens (mesh size is shown for second grinding), to determine the effect of relative particle size on ß-glucan determination (Marion oats). Values represent the mean ± standard deviation (n=3).

Sample	Screen Mesh (nominal)	% ß-Glucan
1-0	1.1mm	5.3±.09
1-1	1.1mm	5.9±.10
1-2	0.5mm	5.3± 0
1-3	0.4mm	5.8±.11
1-4	0.2mm	4.7±.03
1-5	0.1mm	3.7±.02
1-6	0.08mm	3.3±.02

3.3.1.2 ß-Glucan content of Canadian oats determined enzymatically and by Flow Injection Analysis

Enzymatic determination of ß-glucan content: The ß-glucan contents of a number of registered and unregistered Canadian domestic cultivars of oats, as determined by the modified enzymatic method, is shown in Table 4. The difference in ß-glucan content between the highest and lowest cultivar for a given location in a given year in the present study was 1.5-2.0%, depending on location and year. Marion always gave the highest ß-glucan value, with the maximum (6.3%) observed in the sample grown in Ste. Rosalie in 1986. OA516-2 (3.6%, Charlottetown, 1984) and Donald (3.5%, Ottawa, 1986) represent the low end of the range. In general, the differences in ß-glucan content of the cultivars in between the two extremes were much smaller, although the rank order of the cultivars was consistent.

The range of ß-glucan contents observed in this study is similar to ranges reported in European oats (3.2-6.3%, Welch and Lloyd 1989), and in some Canadian cultivars as well (3.91-6.82%, Wood *et al* 1991b). A lower range was reported in a survey of Swedish oats (2.2-4.2%); these workers, however, used covered oats rather than groats for their analyses (Åman and Graham 1987b), with the result that the ß-glucan content was diluted by hull material.

Assay of ß-glucan by Flow Injection Analysis: Although the basis for the determination of ß-glucan is different for Flow Injection Analysis (FIA) than for the enzymatic method, good agreement has been reported between the two methods for both barley (Jorgensen 1988; Jorgensen and Aastrup 1988a; Sendra *et al* 1989; Switala *et al* 1989; Anderson 1990) and oats (Åman and Graham 1987b). As the FIA method is faster and simpler to perform than the enzymatic method, the samples from this study

Table 4. β-Glucan content of Canadian cultivars of oats. β-Glucan content (%, dry weight basis, groats) measured using the modified enzymatic method for oats grown at 5 locations in eastern Canada over 3 years. Each value represents the mean of 3 determinations.

Cultivar	Year	Location				
		OT	KK	CT	AC	SR
Shaw	1984	4.7	4.2	4.5	4.6	5.2
Tibor	"	4.6	4.3	4.2	4.6	4.7
Ogle	"	5.2	4.5	4.6	4.9	4.6
Woodstock	"	4.83	4.0	4.4	4.4	4.6
Marion	"	N/A	5.6	5.3	5.9	6.0
OA447-27	"	4.9	4.4	4.3	4.7	4.9
OA516-2	"	4.3	3.8	3.6	3.7	4.2
QO191.70	"	5.7	4.3	4.6	4.7	5.2
QO199.27	"	5.1	4.0	4.4	4.4	5.1
QO199.60	"	5.3	4.1	4.3	4.4	5.0
Shaw	1985	5.3	N/A	5.0	4.6	4.6
Ogle	"	5.2	"	5.0	4.5	4.8
Woodstock	"	4.7	"	4.7	4.4	4.8
Marion	"	5.5	"	5.7	5.7	5.8
OA447-27	"	5.1	"	4.8	4.6	4.6
OA516-2	"	3.8	"	4.3	3.8	4.0
QO191.70	"	5.6	"	5.3	4.8	5.0
QO199.27	"	5.0	"	5.1	4.4	4.8
QO199.60	"	5.0	"	5.2	4.3	4.8
Tibor	1986	4.3	4.7	5.1	4.5	4.8
Woodstock	"	4.3	5.0	4.8	4.9	4.6
Marion	"	5.4	6.3	6.1	6.2	6.3
Donald	"	3.5	4.0	4.1	4.4	4.6
QO191.70	"	4.7	5.5	5.2	5.4	4.8
QO199.60	"	5.2	5.2	5.5	5.2	5.5
QO220.9	"	4.9	5.4	5.8	5.4	5.0
QO220.13	"	5.0	5.2	5.5	5.0	5.0

N/A; not available. Locations: OT, Ottawa; KK, Kapuskasing; CT, Charlottetown; AC, Ailsa Craig; SR, Ste. Rosalie.

were analyzed by FIA using the procedure of Jorgensen (Jorgensen 1988, Jorgensen and Aastrup 1988), and the results (Table 5) compared with those obtained using the enzymatic method.

When the ß-glucan values obtained for each cultivar using the two methods were compared overall, there was a high positive association (r=0.90, Fig. 7). When compared for specific cultivars, however, the association varied considerably. In general, the ranking of cultivars with respect to ß-glucan content was the same regardless of the method used (with Marion and OA516-2 as the high and low extremes), but the values obtained using FIA tended to be slightly lower.

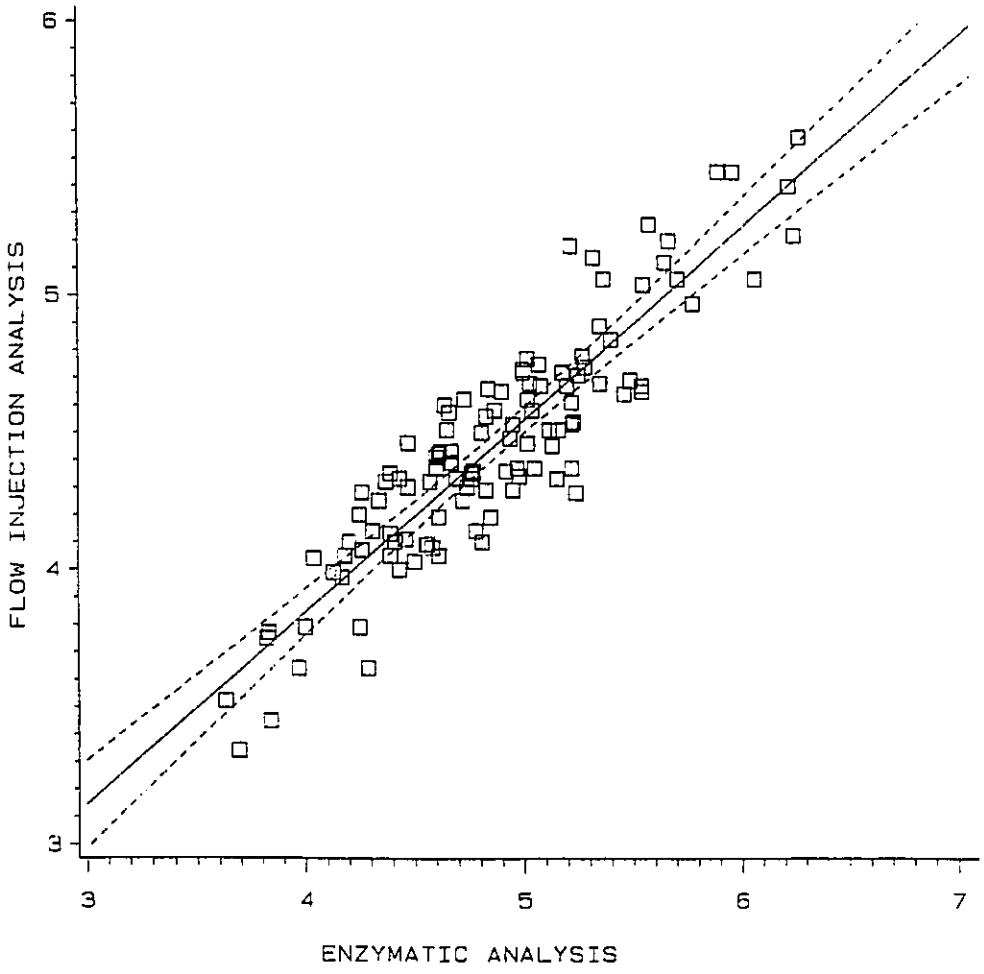
This difference may be accounted for if a small amount of hydrolysis of ß-glucan occurred in the second step of the extraction process for FIA, which is carried out in weak sulfuric acid (37.5 mM). Jorgensen and Aastrup (1988a) showed that there was, in fact, a shift in the molecular weight distribution of ß-glucan in barley samples during the acid extraction step. Foldager and Jorgensen (1984) reported that the increase in Calcofluor fluorescence intensity upon which the FIA method is based occurred only with ß-glucan molecules which were larger than 10^4 daltons. Anderson (1990) found that the FIA method was optimally sensitive to ß-glucans of molecular weight greater than $5x10^4$, and that sensitivity declined to less than 5% of the maximum with ß-glucans smaller than 10^4 daltons. Determination of ß-glucans of differing molecular weights (produced by enzymatic hydrolysis) using FIA was also reported by Manzanares *et al* (1991). In that study, in low ionic strength buffer, all of the ß-glucan of molecular weight greater than $2x10^5$ Da was detected, 50% of the ß-glucan of molecular weight $4.9x10^4$ Da was detected, and only 10% of molecules $2.3x10^4$ Da were detected. Increasing the ionic strength of the buffer by increasing NaCl concentration up to 1% enabled detection of

Table 5. ß-Glucan content measured by Flow Injection Analysis. ß-Glucan content (%, dry weight basis, groats) of oats grown at 5 locations in eastern Canada over 3 years determined using Flow Injection Analysis. Each value represents the mean of 2 determinations.

Cultivar	Year	Location				
		OT	KK	CT	AC	SR
Shaw	1984	4.3	4.0	4.3	N/A	4.7
Tibor	"	4.2	4.2	4.1	4.1	4.4
Ogle	"	4.3	4.5	4.4	4.6	4.6
Woodstock	"	4.6	4.0	4.4	4.1	4.1
Marion	"	N/A	5.3	5.1	5.5	5.5
OA447-27	"	4.4	4.3	N/A	4.3	4.7
OA516-2	"	3.8	3.8	3.5	3.3	4.1
QO191.70	"	5.1	4.3	4.4	4.6	5.2
QO199.27	"	4.7	3.8	4.3	N/A	4.8
QO199.60	"	4.7	4.0	N/A	4.1	4.6
Shaw	1985	4.7	N/A	4.5	4.1	4.4
Ogle	"	4.7	"	4.7	4.0	4.4
Woodstock	"	4.5	"	4.4	4.0	4.3
Marion	"	N/A	"	5.2	5.1	N/A
OA447-27	"	4.5	"	4.4	4.1	4.4
OA516-2	"	3.8	"	4.1	3.5	3.6
QO191.70	"	5.0	"	4.8	4.1	4.7
QO199.27	"	4.8	"	4.5	4.1	4.7
QO199.60	"	4.5	"	4.5	4.3	4.5
Tibor	1986	3.6	4.3	4.4	4.1	4.3
Woodstock	"	4.1	4.4	4.1	4.2	4.3
Marion	"	5.1	5.2	5.1	5.4	5.6
QO191.70	"	4.6	4.7	4.5	4.7	4.4
QO199.60	"	4.3	4.6	4.7	4.4	4.6
QO220.9	"	4.5	4.9	5.0	4.8	4.7
QO220.13	"	4.6	4.5	4.7	4.3	4.3

N/A, not available. Locations: OT, Ottawa; KK, Kapuskasing; CT, Charlottetown; AC, Ailsa Craig; SR, Ste. Rosalie.

Figure 7. Comparison of results of the enzymatic method and the flow injection analysis method for determination of β-glucan in oats. The regression line is represented by the solid line, and the 95% confidence limits are shown as dotted lines. (r=0.90)


FLOW INJECTION ANALYSIS
ENZYMATIC ANALYSIS
3
4
5
6
7

lower molecular weight ß-glucans: all of the ß-glucan of $5.5x10^4$ Da or greater was detected, 50% of ß-glucan of $2.1x10^4$ Da was detected, and 10% of the ß-glucan of molecular weight 10^4 was detected. In the present work, if hydrolysis during the acid extraction step occurred to the extent that some of the ß-glucan molecules were in the smaller molecular weight ranges where incomplete detection with Calcofluor has been reported, slightly lower values for ß-glucan would be expected from the FIA method. The enzymatic method does not distinguish between different sized molecules, and thus would measure all of the ß-glucan in the sample.

From the data presented, it is concluded that FIA would be a useful screening tool to investigate relative amounts of ß-glucan in different cultivars of oats. The specificity of the enzymatic assay, however, coupled with the higher values obtained over a range of samples, appears to provide a more accurate measure of the total ß-glucan in oats.

3.3.2 Variation in ß-glucan content

3.3.2.1 Variation within *Avena sativa*:

Statistical analyses were performed on the data obtained using the enzymatic method, as this was considered to be a more accurate estimate of total ß-glucan. Analysis of variance indicated that the greater source of variation in ß-glucan content was genetic, rather than environmental. Table 6 shows the effect of location, cultivar and location by cultivar (locationXcultivar) interaction on ß-glucan content in oats for each of the three years included in the study. In addition to significant cultivar (genetic) and location (environmental) effects, the locationXcultivar interaction was also significant, indicating that the effect of location was different for each cultivar. The effect of the locationXcultivar interaction was much smaller than either the genetic or environmental

Table 6. Effect of location, cultivar and locationXcultivar interaction on the ß-glucan content of oats expressed as an F-value in the two-way analysis of variance in three separate growing seasons (1984, 1985, 1986).

Year	Source of Variation	DF	F-Value
1984	location	4	309.57**
	cultivar	9	322.25**
	locationXcultivar	35	22.32**
	Test of hypothesis using locationXcultivar as error term:		
	location	4	13.83**
	cultivar	9	14.63**
1985	location	3	126.35**
	cultivar	8	304.75**
	locationXcultivar	23	18.82**
	Test of hypothesis using locationXcultivar as error term:		
	location	3	6.71**
	cultivar	8	16.20**
1986	location	4	101.02**
	cultivar	7	423.91**
	locationXcultivar	27	13.91**
	Test of hypothesis using locationXcultivar as error term:		
	location	4	7.26**
	cultivar	7	30.43**

DF, degrees of freedom
** significant effect at $\alpha = .01$

influences. When the analysis of variance was repeated using the locationXcultivar interaction as an error term, thus effectively removing the influence of the interaction, the genetic influence on ß-glucan content was clearly greater than the environmental effect for each year of the study (α=.01), although the magnitude of the difference varied from year to year.

Greater genetic than environmental variation has also been reported both for specific crossings of barleys (Greenberg 1977), and for larger scale screenings of commercial and breeder's lines of barley (Aastrup 1979; Gill *et al* 1982; Molino-Cano and Conde 1982; Bourne and Wheeler 1984; Åman 1986; Henry 1986; Palmer and MacKenzie 1986; Lehtonen and Aikasalo 1987; Truelsen 1987), and for some lines of oats as well (Åman and Graham 1987b).

The values shown in Table 7 represent mean ß-glucan values for each oat cultivar over all locations for each year. Duncan's Multiple Range Test (α=.05) was helpful in identifying real differences in ß-glucan content among the cultivars which were not necessarily apparent from the mean values (Table 7). In all three years, Marion was clearly higher than the other cultivars surveyed, and was the sole occupant of its Duncan grouping. In 1984 and 1985, the two years in which OA516-2 was included in the test, this cultivar was alone in its grouping at the bottom of the range. The cultivars with ß-glucan contents in between the two extremes for these years show some differences, but the Duncan groupings show considerable overlap, so that only the highest and lowest cultivars within this range can truly be considered different. In 1986, the differences between cultivars were clearer, with no overlap between groupings. Overall, the value for the highest cultivar (Marion) was about 30% greater than the value for the lowest cultivar (OA516-2 for 1984 and 1985, Donald for 1986). In general, the ß-glucan contents of the

Table 7. Amount of ß-glucan (% dry weight basis, groats) in oat cultivars over 3 years (Mean ± Standard Error). For 1984 and 1986, the value represents the means of three determinations at each of 5 locations. For 1985, the material was available for 4 locations only. Values in each year with the same superscript are not significantly different from each other (Duncan's Multiple Range test, $\alpha = .05$)

Cultivar	1984	1985	1986
Shaw	$4.7 \pm .09^{bc}$	$4.8 \pm .09^{cd}$	
Tibor	$4.5 \pm .06^{c}$		$4.7 \pm .08^{c}$
Ogle	$4.7 \pm .07^{bc}$	$4.9 \pm .09^{c}$	
Woodstock	$4.5 \pm .08^{c}$	$4.6 \pm .05^{d}$	$4.7 \pm .07^{c}$
Marion	$5.7 \pm .08^{a}$	$5.7 \pm .04^{a}$	$6.0 \pm .10^{a}$
Donald			$4.1 \pm .10^{d}$
OA447-27	$4.6 \pm .08^{bc}$	$4.8 \pm .07^{cd}$	
OA516-2	$3.9 \pm .07^{d}$	$4.0 \pm .06^{e}$	
QO191.70	$4.9 \pm .14^{b}$	$5.2 \pm .09^{b}$	$5.1 \pm .09^{b}$
QO199.27	$4.6 \pm .12^{bc}$	$4.9 \pm .09^{bc}$	
QO199.60	$4.6 \pm .12^{bc}$	$4.8 \pm .10^{cd}$	$5.3 \pm .04^{b}$
QO220.9			$5.3 \pm .09^{b}$
QO220.13			$5.1 \pm .06^{b}$

cultivars in between the two extremes were much closer together, and the differences did not always reach significance, although the rank order of the cultivars was consistent (confidence level of 98%). In a smaller study, which did not encompass environmental effects, Wood *et al* (1991b) observed a similar ranking in the cultivars Marion, Woodstock, Ogle, Tibor, Donald, and OA516-2.

Two sets of iso-lines (progeny from the same crosses) were included in this study: QO199.27 and QO199.60; and QO220.9 and QO220.13. These pairs are essentially indistinguishable in terms of ß-glucan content, and also with respect to other characteristics measured.

Effect of precipitation and temperature: Using data from the cultivars Marion, Woodstock, QO191.70 and QO199.60, which were grown in almost all locations for all the years, an attempt was made to determine the relative effects of precipitation and temperature on ß-glucan content. The mean temperatures and total cumulative precipitation from seeding date to harvest (Table 8) were used in these calculations. Analysis of the data by individual cultivar, or pooled over all four cultivars yields similar results. A summary of the general effects attributable to precipitation and temperature is given in Table 9. These figures indicate that there was a low negative association between ß-glucan content and precipitation (r=-.40), but the unexplained component (that component of variation which was not related to precipitation) was very high (84%). There was an even weaker positive association between temperature and ß-glucan content (r=.26), and here the unexplained component was even higher (93%). These results indicate that precipitation and temperature are not dominant factors influencing

Table 8. Summary of environmental conditions. Mean temperature (Temp.) and total cumulative precipitation (Precip.) for growing seasons in 1984, 1985 and 1986 for the five locations included in the study.

Location	1984		1985		1986	
	Temp. °C	Precip. mm	Temp. °C	Precip. mm	Temp. °C	Precip. mm
Ottawa	18.8	384.6	17.4	274.9	17.1	522.8
Kapuskasing	13.5	421.3			13.4	264.9
Charlottetown	17.0	414.4	16.6	327.8	14.6	352.0
Ailsa Craig	17.5	477.0	17.0	420.2	16.8	346.4
Ste. Rosalie	18.1	420.9	17.4	287.0	18.6	393.1

Table 9. Effect of environment on ß-glucan content. A summary of the effects attributable to precipitation and temperature on ß-glucan content of oats. Pearson correlation coefficient, r; coefficient of determination, r^2; P, probability; Conf., confidence; and N, number of observations.

	r	r^2	P	Conf.	N
Precip	-.40	0.16	.005	.99	54
Temp	.26	0.07	.05	.90	54

ß-glucan levels in oats. A negative relationship between precipitation and ß-glucan content was previously reported in barley (Aastrup 1979, Coles 1979).

3.3.2.2 Variation among different species of *Avena*

The range of ß-glucan content observed in this study, and reported elsewhere in commercial cultivars of *A. sativa* is relatively narrow, with the lowest values for groats being 3-3.5%, and the highest values levelling off at 6-6.5% depending on location and growing season (Henry 1985; Åman and Graham 1987b; Welch and Lloyd 1989; Wood *et al* 1991b). To determine the feasibility of extending the range of ß-glucan contents available for breeding purposes, and thus potentially better serve the disparate requirements of human and livestock nutrition, the ß-glucan content of 18 primitive species of *Avena* was determined, along with a selection of domestic cultivars grown in the same field in the same season as the primitives. These domestic cultivars represented the range of ß-glucan contents observed for Canadian cultivars (Table 10). The ß-glucan content in the primitive species studied ranged between 1.8% to 5.5%, with significant differences between species (Duncans's Multiple Range Test, α=.05). The lower limit of this range is well below the level of the low ß-glucan domestic cultivars grown in the same experiment for comparison (Tibor, 3.7%; and OA516-2, 3.8%). The upper limit of ß-glucan content in the 18 species studied (*A. hirtula*, 5.5%), although significantly higher, was much closer to the domestic cultivar with the highest ß-glucan content reported to date (Marion, 5.0% in this growing season). The domestic cultivars separated into groupings similar to those already observed for Canadian oats.

The diploids, which contain the species most distantly related to *A. sativa* (Holden 1976), contained the species with the lowest (*A.eriantha*, 1.8%) but also the highest

Table 10. Mixed linkage ß-glucan and protein content (%, dry weight basis, groats) and thousand kernel weight (TKW) of different species of *Avena*. Values for ß-glucan content represent the mean (n=3) ± standard deviation. Values for protein content are means of duplicate determinations. TKW were calculated from the weight of 150 seeds. Numbers in each column with the same superscript are not significantly different.

Species	% ß-Glucan	% Protein	TKW(g)
DIPLOIDS			
A.eriantha	1.8 ±.10^{l}	30.7^{d}	4.7
A.ventricosa	2.3 ±.07^{k}	33.7^{a}	5.0
A.canariensis	2.8 ±.04^{j}	23.3^{k}	11.4
A.pilosa	2.9 ±.03^{ij}	33.1^{b}	4.0
A.longiglumis	3.1 ±.07^{gh}	31.3^{c}	9.0
A.clauda	3.2 ±.01^{g}	33.0^{b}	3.5
A.strigosa	4.6 ±.03^{d}	20.0^{p}	18.5
A.lusitanica	5.1 ±.07^{bc}	26.9^{h}	8.7
A.hirtula	5.5 ±.09^{a}	30.2^{e}	3.3
MEAN	3.5^{B}	29.1^{A}	7.6^{C}
TETRAPLOIDS			
A.magna	2.4 ±.18^{k}	29.9^{f}	32.1
A.murphyi	3.0 ±.04^{hi}	28.2^{g}	30.7
A.barbata	3.7 ±.13^{f}	28.4^{g}	5.7
A.vaviloviana	3.9 ±.07^{f}	23.0^{k}	9.4
MEAN	3.2^{B}	27.4^{AB}	19.5^{B}
HEXAPLOIDS			
A.occidentalis	4.3 ±.08^{e}	24.2^{ij}	11.9
A.fatua	4.5 ±.03^{d}	22.6^{l}	17.9
A.byzantina	5.0 ±.10^{c}	21.6^{n}	24.4
A.sterilis	5.2 ±.13^{bc}	24.4^{i}	12.6
A.hybrida	5.3 ±.13^{b}	23.9^{j}	11.5
MEAN	4.9^{A}	23.3^{BC}	15.7^{BC}
A.sativa (domestic)			
Tibor	3.7 ±.16^{f}	21.9^{mn}	31.7
OA516-2	3.8 ±.11^{f}	21.7^{mn}	31.5
Woodstock	4.1 ±.05^{e}	22.1^{m}	28.9
Donald	4.2 ±.10^{e}	16.7^{q}	31.5
Marion	5.0 ±.12^{c}	21.2^{o}	36.1
MEAN	4.2^{AB}	20.7^{C}	31.9^{A}

(*A.hirtula*, 5.5%) levels of ß-glucan. This group also contained the greatest number of species (9). The tetraploids, a much smaller group (only 4 species), had a much narrower range: from 2.4% (*A. magna*) to 3.9% (*A. vaviloviana*). The primitive hexaploids (5 species) also had a narrow range relative to the diploids: from 4.3% (*A. occidentalis*) to 5.3% ß-glucan (*A. hybrida*). The domestic hexaploids (5 cultivars), which were considered separately because they all belong to the same species (*A. sativa*), had a wider range than the primitive hexaploids: 3.7% (Tibor) to 5.0% (Marion). Within the primitive groups, the ß-glucan contents of most of the species were significantly different from each other (Table 10, lower case superscripts).

In addition to differences between species, when the means for each group were compared, Duncan's Multiple Range Test (α=.05) shows that there were significant differences in ß-glucan content with respect to ploidy level (Table 10, upper case superscripts). The tetraploids, as a group, had a significantly lower ß-glucan content (3.24%) than the primitive hexaploids (4.9%). The mean ß-glucan content for the 5 domestic cultivars (4.2%) was not significantly different from the primitive hexaploids. The broad range found in the diploids puts this group in the middle with a mean ß-glucan content of 3.5%, which is not significantly different from the means for either the tetraploids or domestic hexaploids. The mean ß-glucan content for the primitive hexaploids was significantly higher than that for the diploids. The significance of the differences in ß-glucan content with respect to ploidy is unclear at this time, as the data is limited to only 18 species, represented by a single variety for each. The potential for breeding, however, is limited, as the group with the greatest range of ß-glucan contents (the diploids) is the most difficult group to cross with the domestic cultivars.

The range of ß-glucan contents observed in the present study is similar to the

range reported in a study of eight species of greenhouse-grown primitive oats (1.2-5.7%, Welch *et al* 1991), which included 4 diploids, 3 tetraploids and a hexaploid. Although the absolute values for ß-glucan content for the eight species reported by Welch *et al* (1991) were different for the same species in the present study, the ranking of the species was similar for the two studies. No relationship was found between ploidy level and kernel ß-glucan (Welch *et al* 1991).

3.3.2.3 Relationship between ß-glucan and quality parameters in oats

Of the cereals, oats have one of the highest protein contents, and the protein in oats is considered to be of higher quality, by virtue of having higher levels of essential amino acids that are typically limiting in cereals (Burrows 1986; Lockhart and Hurt 1986; Peterson and Brinegar 1986). Oat groats also have the highest lipid concentration among the cereal grains (Youngs 1986). If the information obtained regarding the genetic variability of ß-glucan content is to be useful from a breeding standpoint, it should be viewed in the context of other kernel characteristics, such as protein and oil. To address all of the parameters considered in an established oat breeding program is well beyond the scope of this thesis. Nevertheless, some information on protein and oil content (measured on a whole oat basis) was available for the samples from the Eastern Co-operative Oat Test. These values were converted to a groat basis using hull% data (not shown) measured on the samples available (Tables 11 and 12 respectively). Protein contents were also determined for the primitive species grown (measured on a groat basis, Table 10), and thousand kernel weights (TKW) were measured for both primitive and domestic samples (Tables 10 and 13 respectively).

Table 11. Protein content (%) of Canadian cultivars of oats, on a groat basis. For 1984 and 1986, the value represents the means of duplicate determinations at each of 5 locations ± standard error. For 1985, material was available for 4 locations only.

Cultivar	1984	1985	1986
Shaw	14.9±0.5	15.8±1.1	
Tibor	17.4±0.7		18.3±0.3
Ogle	15.1±0.6	15.6±0.8	
Woodstock	15.5±0.6	17.7±1.3	18.1±0.7
Marion	15.2±0.4	15.8±1.0	16.7±0.7
Donald			16.5±0.5
OA447-27	16.0±0.6	16.2±0.8	
OA516-2	15.2±0.7	15.4±1.0	
QO191.70	14.6±0.5	15.3±1.0	16.7±0.6
QO199.27	14.5±0.5	15.2±0.9	
QO199.60	14.3±0.5	14.6±0.8	17.3±0.6
QO220.9			16.6±0.7
QO220.13			16.3±0.7

Table 12. Oil content (%, groat basis) of Canadian oats. For 1984 and 1986, the value represents the mean ± standard error for 5 locations. For 1985, material was available for 4 locations only.

Cultivar	1984	1985	1986
Shaw	8.0±0.4	8.2±0.2	
Tibor	7.4±0.3		6.4±0.2
Ogle	5.8±0.2	6.1±0.1	
Woodstock	7.3±0.3	7.7±0.2	6.8±0.3
Marion	7.5±0.4	7.9±0.3	7.2±0.4
Donald			5.7±0.3
OA447-27	6.0±0.2	5.9±0.2	
OA516-2	7.8±0.3	7.7±0.3	
QO191.70	6.0±0.3	6.1±0.0	6.0±0.2
QO199.27	6.1±0.3	6.1±0.2	
QO199.60	6.3±0.3	6.0±0.1	6.2±0.1
QO220.9			6.7±0.1
QO220.13			7.8±0.3

Table 13. Thousand kernel weights (g) for Canadian oats, determined on a groat basis (calculated from the weight of 150 kernels). For 1984 and 1986, the value represents the means for 5 locations (n=5) ± standard error. For 1985, material was available for 4 locations only (n=4).

Cultivar	1984	1985	1986
Shaw	23.9±1.3	22.9±1.5	
Tibor	27.0±0.2		27.6±1.9
Ogle	23.1±1.5	22.1±1.6	
Woodstock	23.1±0.6	21.4±1.7	21.5±1.2
Marion	26.2±1.8	25.1±2.0	25.0±1.6
Donald			25.1±2.0
OA447-27	27.9±1.7	28.4±0.4	
OA516-2	26.7±2.5	28.4±2.0	
QO191.70	23.9±1.6	23.5±0.4	21.9±1.9
QO199.27	23.3±1.1	22.0±1.7	
QO199.60	22.8±1.5	21.9±0.4	21.8±1.8
QO220.9			22.8±1.5
QO220.13			22.3±1.8

When the domestic cultivars were considered individually, over all of the locations and years of the study, the association between ß-glucan and groat protein content was positive, although variable between cultivars. The highest association was found for QO199.60 (r= 0.75), and the lowest was Marion (r= 0.24). When compared over all of the cultivars, for all of the locations and years of the study, the positive association between ß-glucan and protein content was very weak (r=0.27), indicating that these characteristics are not related. This is in agreement with previous reports suggesting that within individual cultivars there was an association between ß-glucan and protein, but overall (100 genotypes), no significant correlation existed between the two characteristics (Welch and Lloyd 1989; Welch *et al* 1991).

With the exception of 3 cultivars, comparison of ß-glucan content with oil content by cultivar showed a negative association which was variable between cultivars. The highest negative association was found for OA447-27 (r=-0.80), and the lowest was Woodstock (r=-0.31). The exceptions (Donald, QO220.9 and QO220.13) also showed variation in the strength of the positive association (r=0.84, 0.49 and 0.73 respectively). When compared over all of the cultivars, for all of the locations and years of the study, a very weak negative association was found between ß-glucan content and oil content (r=-0.10), indicating that the two characteristics are not related. Both oil and protein content are subject to environmental influences, as is ß-glucan content (Burrows 1986), and the associations observed could reflect some of those influences.

The protein content of the 18 primitive species of *Avena* as well as the domestics grown at the same time for comparison were assayed as % nitrogen, and converted to protein values using a factor of 6.25 (Table 10). The levels of protein determined varied between species, and in general were much higher in the primitive species than in the

domestic cultivars. Mean protein levels in the diploids (29.1%) and tetraploids (27.4%) were not significantly different from each other, but they were significantly higher than the domestics (20.7%, Duncan's Multiple Range Test, $\alpha=.05$). The primitive hexaploids had a higher mean protein content (23.3%) than the domestics, but this difference did not reach significance at $\alpha=.05$. The protein contents observed for primitive oats in the present study are similar to those reported by Welch *et al* (1991) for 8 species of primitive oats, although the ranking of the species was slightly different in the two studies. This may reflect differences in greenhouse- and field-grown material.

When the domestic oat cultivars from the genetic/environmental study are considered separately, there was some indication that, in some cultivars, TKW and ß-glucan had an inverse relationship. When compared by location, or by mean levels for the cultivars covered generally throughout the study, however, there was no provable association between TKW and ß-glucan content, suggesting that these two characteristics are independent. No association was found between cultivar yield (kg/hectare, data not shown) and ß-glucan content in the domestic cultivars.

When compared with other species of *Avena*, the domestic cultivars, with a mean TKW of 31.9 g, were significantly higher than all of the other groups (Table 10). In the domestic hexaploids, which are all *A. sativa* the range of TKWs is predictably much narrower (7.2 g) than the ranges for the other groups (diploids 15.2 g, tetraploids 26.4 g, primitive hexaploids 12.9 g) which are composed of different species of *Avena*. The tetraploids (mean TKW of 19.5 g) and primitive hexaploids (mean TKW 15.7 g) were not found to be significantly different from each other. The diploids, with a mean TKW of 7.6 g, are the lowest group. Because of the broad range observed in the diploids, however, the mean was not found to be significantly different ($\alpha=.05$) from the primitive hexaploids.

The success of breeders in improving the seed size for commercial oats is amply illustrated by this data.

When compared over all of the domestic cultivars for all of the locations over all three years of the genetic and environmental study, there was a very weak negative correlation between protein and TKW (r=-0.12), suggesting that these two characteristics are not related. When compared in different species as well as selected cultivars, however (Table 10), a stronger negative correlation was observed (r=-0.63). No relationship was found between lipid and TKW (r=0.17) or between protein and lipid (r=0.09) in the domestic cultivars.

The range of ß-glucan contents currently available in domestic cultivars in Canada offers the potential for producers to grow oats for specific markets. For human nutrition, where soluble fibre from oats (which is composed primarily of ß-glucan) has been reported to improve lipid and carbohydrate metabolism (Kirby *et al* 1981; Anderson and Chen 1986, Anderson and Gustafson 1988; Gold and Davidson 1988; Wood *et al* 1989, Anderson *et al* 1990; Demark-Wahnefried *et al* 1990; Wood *et al* 1990; Van Horn *et al* 1991; Davidson *et al* 1991), a high ß-glucan cultivar such as Marion would be desirable. In contrast to the reported clinical benefits in human nutrition, high levels of ß-glucan in livestock diets have been shown to result in low feed conversion efficiencies and lower, slower weight gains than other diets, particularly in poultry (Potter *et al* 1965, Hesselman *et al* 1982, Cave *et al* 1990). For purposes of livestock feeding, low ß-glucan cultivars such as OA516-2 (which is not yet licensed) or Donald (which is a licensed cultivar) would be desirable. In addition to low levels of ß-glucan, OA516-2 contains relatively high levels of protein (Table 11) and lipid (Table 12), which would increase its value as animal feed.

The high protein contents and wider range of ß-glucan contents observed in the

primitive oats are useful qualities for inclusion in a breeding program. With the exception of *A. magna* and *A. murphyi*, however, these attributes are accompanied by much smaller seed sizes, as indicated by TKW, than commercial cultivars. In addition, interspecific crossing is a difficult and time consuming process (V.D. Burrows, personal communication). In light of these considerations, the possibilities for extending the high and low limits of ß-glucan content in domestic oats by interspecific crossings with some of the primitive species appear to be limited.

Although the range of ß-glucan contents observed in the primitive species was somewhat wider than the range seen in the domestic cultivars, the range extension in the present study is restricted to the lower end. In barley, low ß-glucan content has been reported to be a dominant characteristic (Greenberg 1977), although additive effects were also found to be important (Lance 1984). If ß-glucan inheritance is similar in oats, breeding a lower or higher ß-glucan oat could be more efficiently achieved by appropriate crosses using hexaploid material from domestic *A. sativa* after screening for the desired characteristics. The use of mutagens to produce low ß-glucan mutants in barley has also been reported (Aastrup 1983, Aastrup and Munck 1985), and may find application in oats as well.

3.3.2.4 Variation in molecular Structure of ß-glucan

Trisaccharide (3-O-cellobiosyl-D-glucose) and tetrasaccharide (3-O-cellotriosyl-D-glucose) account for 90% of the oligosaccharides released by lichenase digestion of oat (Wood *et al* 1991a) and barley (Woodward *et al* 1983b) ß-glucan, and thus represent the major structural repeating units of the ß-glucan. The ratio of trisaccharide:tetrasaccharide varies in ß-glucans from different sources in both vegetative and reproductive tissues

(Nevins *et al* 1978; Stinard and Nevins 1980; Wood *et al* 1991a), and is a useful indicator of structural variation in ß-glucan. Because of the specificity of lichenase, this ratio can be determined in samples without having to purify the polysaccharide (Staudte *et al* 1983; Wood *et al* 1991a).

The molar ratios of tri-:tetrasaccharides observed in both domestic and primitive oats are shown in Table 14. In addition to tri- and tetrasaccharides, larger oligosaccharides (degree of polymerization 5 and 6) were visible in some cases, but the peaks were very small, and generally these fractions were lost in the dilution required to analyze the tri- and tetrasaccharides. Samples include a group of domestic cultivars grown in Ste. Rosalie in 1985 (cultivars Marion, Ogle, Shaw, Woodstock, OA447-27, OA516-2, QO191.70, QO199.27 and QO199.60) and the 18 primitive species of *Avena*, plus 5 domestic cultivars (Donald, Marion, Ogle, Tibor and Woodstock) grown in Ottawa in 1988. The range of molar ratios observed in the 32 samples measured was 1.81-2.33. Duncan's Multiple Range Test (α=.05) places these samples into 10 groups (a-j). Although many of these groups show considerable overlap, significant differences in molar ratios of tri-:tetrasaccharides can be seen between some species. The range of molar ratios in the domestic cultivars alone was from 2.05-2.22, and although the ratios at the two extremes are significantly different, in general, the ratios in between are not. This range is in agreement with the data of Wood *et al* (1991a), who reported range of molar ratios from 2.09-2.25 in oats. In all three cases where the same cultivar was grown in both locations in the different growing seasons, the molar ratios were not significantly different from each other, suggesting that environ-mental conditions are not a major factor in the structural variation of oat ß-glucans. The molar ratios of the iso-lines QO199.27 and

Table 14. Variation in molecular structure of oat ß-glucan. Molar ratios of tri-:tetrasaccharide released by lichenase from domestic oats (*Avena sativa*) grown in Ste. Rosalie in 1985 (*) and in Ottawa in 1988 (/88), and primitive species of *Avena* grown in Ottawa in 1988. Values falling in the same Duncan grouping are not significantly different from each other (n=3).

Species/cultivar	Ratio	Duncan Grouping (α=.05)
A.canariensis	2.33	a
A.longiglumis	2.33	a
A.lusitanica	2.33	a
A.hirtula	2.24	a b
QO199.27*	2.22	b c
Ogle*	2.20	b c d
A.barbata	2.19	b c d e
Ogle/88	2.16	b c d e f
QO199.60*	2.16	b c d e f
A.occidentalis	2.16	b c d e f
A.strigosa	2.15	b c d e f
A.sterilis	2.14	b c d e f
A.ventricosa	2.12	b c d e f g
A.byzantina	2.12	c d e f g
Donald/88	2.11	c d e f g
A.vaviloviana	2.11	c d e f g
A.eriantha	2.11	c d e f g
Tibor/88	2.11	c d e f g
OA516-2*	2.10	c d e f g
A.clauda	2.10	c d e f g
A.hybrida	2.09	d e f g
Woodstock*	2.08	d e f g
Marion*	2.08	d e f g
OA447-27	2.07	e f g
Woodstock/88	2.06	f g h
Shaw*	2.05	f g h
Marion/88	2.05	f g h
QO191.60*	2.05	f g h
A.fatua	2.01	g h
A.magna	1.96	h i
A.pilosa	1.88	i j
A.murphyi	1.81	j

QO199.60 (both grown at the same location in the same season) were also not significantly different from each other.

Three of the cultivars used in the study by Wood *et al* (1991a) were also used in the present study. The ratios found in the present work for these cultivars (Donald, 2.11; Tibor, 2.11; and Marion, 2.05 and 2.08) are in reasonable agreement with the ratios reported by those workers (2.05, 2.19 and 2.07 respectively).

When the domestic cultivars are considered as a group, the range of molar ratios of tri-:tetrasaccharide was narrower (2.05-2.22) than the full range observed for all of the cultivars and species included in the study, and although the ratios for a few cultivars at the two extremes were significantly different, in general, the ratios in between were not. When the primitive species are considered together as groups, no significant differences were observed with respect to ploidy. It is interesting to note, however, that the four species with the highest molar ratios of tri-:tetrasaccharides in group "a" are all diploids. As with ß-glucan content, the range of values for molar ratios is greatest in the diploids, which are the sole occupants of the group with the highest values (a), and there is also a diploid present in the group with the lowest values (j).

Structural differences in ß-glucan in grains as indicated by tri-:tetrasaccharide ratios have been reported between different genera of the cereals. Values are highest in wheat (ratios of 3.04-3.84), followed by barley (2.93-3.41) and rye (2.73) (Wood *et al* 1991a). The results reported here suggest that there are structural differences in ß-glucan within genera as well, although these differences appear to be more subtle.

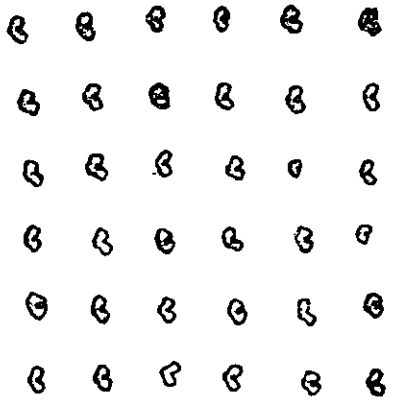
3.3.2.5 Distribution of ß-glucan in caryopses of different cereals

Distribution of ß-glucan in oats: Microscopic evidence has indicated that there are differences in distribution of β-glucan in kernels from different cultivars of oats (Fulcher 1986). Such differences may be related to differences in overall ß-glucan content of the different cultivars, and could also have a profound effect on the processing performance of various cultivars. These differences, however, have not been well characterized to date. The fluorescent dye Calcofluor, which was used to detect extracted ß-glucan in the FIA assay, was also used as a probe for ß-glucan *in situ* to map its distribution in seeds by microspectrofluorometry. In this procedure oat groats were embedded vertically in blocks of polyester resin, and the surface of the block abraded to expose cross sections (Fig. 8) at the desired depth in the seed. The anatomy of a cross section taken through an oat kernel at the proximal end is shown diagramatically in Figure 1. Figure 1 also shows the regions of the kernel which were scanned to map the distribution of ß-glucan.

The relative fluorescence intensity (RFI) of Calcoflour bound to ß-glucans was measured by scanning microspectrofluorometry, and is represented as intensity profiles. The profiles are oriented such that the crease is positioned at the left of the section. The RFI of bound Calcofluor is approximately proportional to the amount of ß-glucan present (Wood and Fulcher 1978; Jensen and Aastrup 1981; Wood and Weisz 1984; Wood 1985; Jorgensen 1988).

Figure 9 shows three intensity profiles representing proximal, central and distal scans of a single kernel of OA516-2, taken from a sample assayed at 4.0% ß-glucan. In the proximal region of the kernel, which contains the embryo (Fig. 9a), the greatest deposition of ß-glucan is in the starchy endosperm immediately adjacent to the embryo (sub-embryo area). When thin sections were stained with Calcofluor and examined

Figure 8. A block of polyester resin containing embedded oat kernels. The block has been abraded to expose cross sections in the central region of the kernels, which have been stained with Calcofluor and Fast Green for scanning.



microscopically (Fig. 10), the cell walls in the sub-embryo area appeared very thick, and little or no cell content was visible. This area is also called the depleted layer (see Fig. 1), and consists of cell walls left behind as the growing embryo encroached on the endosperm during development of the seed (Brown and Morris 1890; Fulcher 1986). The cell walls in the embryo itself were very thin, and contained little β-glucan, as indicated both by the intensity profile and demonstrated by fluorescence microscopy (Fig 10a). A relatively high concentration of ß-glucan is also found in the peripheral regions of the kernel, in the layer of cells just inside the aleurone layer (sub-aleurone layer, see Fig. 1). Examination of thin sections using fluorescence microscopy (Fig. 10b) shows that, as in the depleted layer the cell walls in the sub-aleurone layer were also very thick and that wall thickness decreased in the interior of the starchy endosperm (Fig. 10c,d). Microscopic evidence of thinner walls in the interior of the starchy endosperm was reflected in the lower levels of RFI observed in those areas (Fig. 9a). Differences in cell wall thickness corresponding to differing β-glucan contents have been reported in barleys with different ß-glucan contents (Aastrup 1983).

Moving away from the proximal end into the central region of the kernel, the very large sub-embryo deposition disappeared, and the ß-glucan distribution was similar to that seen in the ventral portion of the section through the proximal region. The ß-glucan content was highest in the sub-aleurone layer (around the periphery of the kernel and up into the crease), with lower levels in the interior of the section. The distribution in the distal portion of the kernel was the same as in the central region.

Small points of fluorescence were also visible outside the kernel at the distal end. These are caused by autofluorescence of the trichomes, or hairs, attached to the kernel. This autofluorescence measured less than 5% RFI, which was the lower threshold set for

Figure 9. Distribution of ß-glucan in a kernel of OA516-2 oats, detected by scanning the relative fluorescence intensity of bound Calcofluor. a) Intensity profile of relative fluorescence intensity at the proximal end of the kernel (containing the embryo); b) Intensity profile of relative fluorescence intensity in the central region of the kernel; c) Intensity profile of relative fluorescence intensity at the proximal end of the kernel.
G, germ; D, depleted layer; C, crease; T, trichomes.

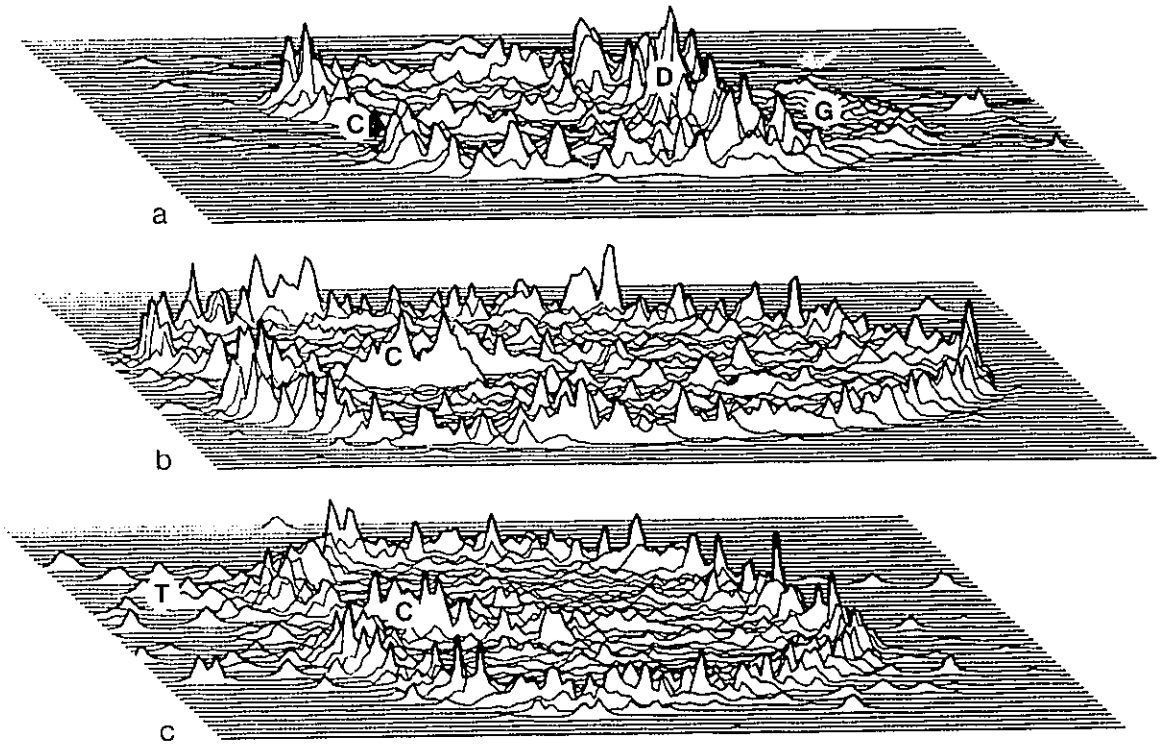
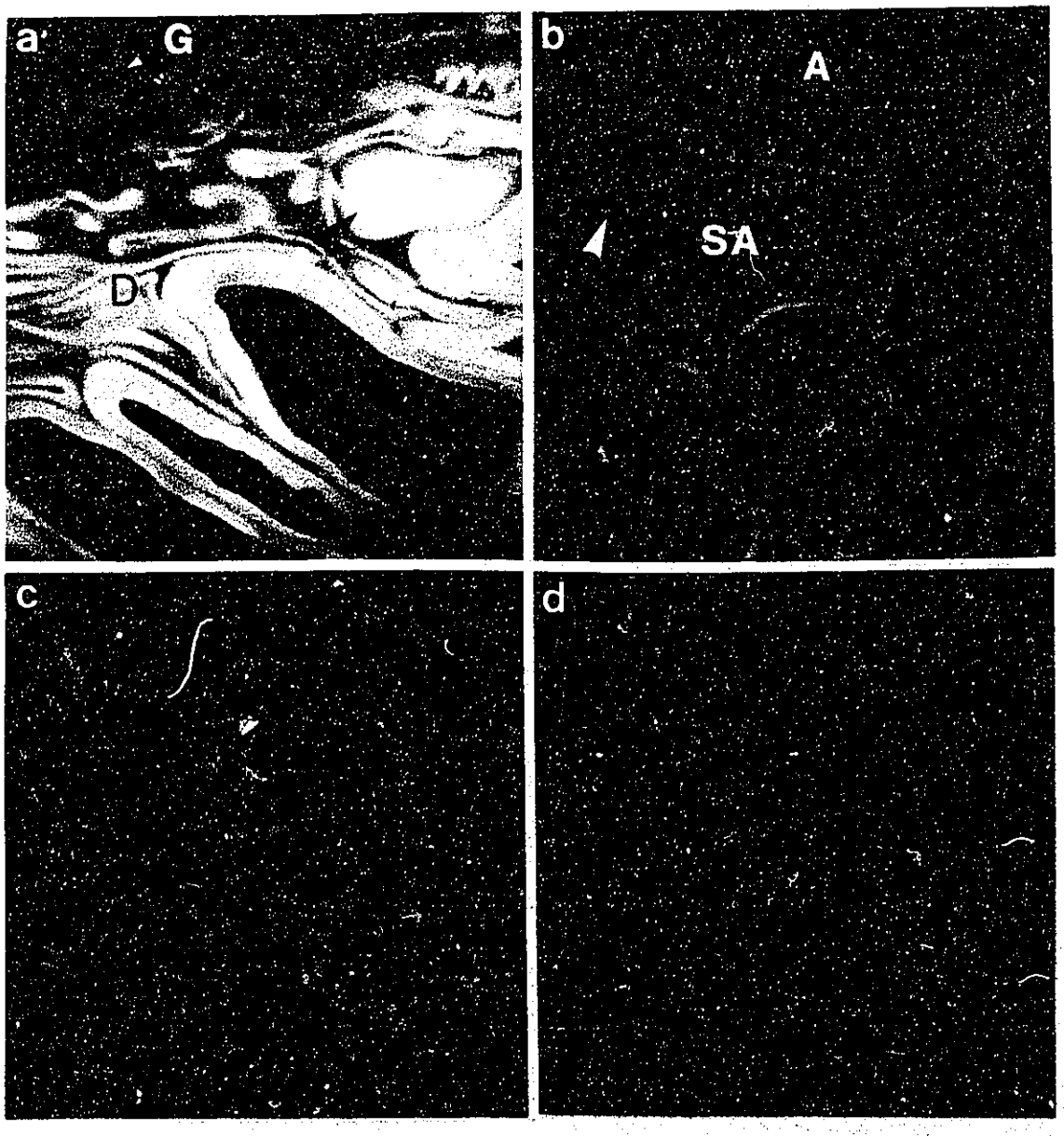

D
C
G
a
C
b
T
C
c

Figure 10. Fluorescence micrographs of sections (2 μm thick) of oats embedded in GMA. Sections were stained with Calcofluor, and counterstained with Fast Green. a) Depleted layer (D) next to the germ (G) in OA516-2 (magnification X40). Note the thick cell walls in the depleted layer (large arrow) and the thinner cell walls in the germ. b) Sub-aleurone layer (SA) and aleurone layer (A) in OA516-2 (magnification X40). Note thick cell walls in the sub-aleurone layer (arrow). c,d) Thin cell walls in the starchy endosperm of OA516-2 (c) and Marion (d) oats (magnification X16).


a
G
D
b
A
SA
c
d

measurements on the microspectrofluorometer, and thus did not interfere with the fluorescence detection of ß-glucan. Phenolics in the aleurone layer (which is found around the periphery of the kernel, see Fig. 1) also autofluoresce at the same excitation wavelength (365 nm) as wall-bound Calcofluor (Fulcher *et al* 1972; Fulcher 1986), as demonstrated in the scan of an unstained oat kernel in Figure 11. The autofluorescence observed in the aleurone cell walls, however, was of much lower intensity than that of wall-bound Calcofluor, and was below 5% RFI which was the low threshold setting used for these experiments. In addition, the autofluorescence in the aleurone layer was quenched to some extent by the Fast Green which was used as a counter-stain in this work. Thus phenolic autofluorescence did not constitute a significant contribution to the fluorescence of bound Calcofluor which was of interest in these samples.

The distribution of fluorescence of wall-bound Calcofluor in a kernel of Woodstock from a sample assayed at 5.1% ß-glucan is presented in Figure 12. Although the RFI throughout the kernel was higher, the pattern of distribution was similar to that in the cultivar OA516-2. In the proximal region (Fig. 12a), the greatest deposition of ß-glucan was adjacent to the embryo. The RFI in the interior of the kernel is somewhat lower than in the sub-aleurone layer, but higher than in the corresponding area in OA516-2. The difference between the sub-aleurone and interior areas of the kernel was less pronounced than in OA516-2. The lowest level of ß-glucan, as in OA516-2, was observed in the embryo. In the central (Fig. 12b) and distal (Fig. 12c) regions of the kernel the pattern was again similar to that observed in OA516-2, with higher ß-glucan depositions in the sub-aleurone layer and smaller amounts in the interior of the endosperm, but the actual values (%RFI) differ.

Figure 11. Distribution of autofluorescence in an unstained oat kernel. a) Profile: plot of distance (x-axis) vs relative fluorescence intensity (y-axis) in 5 scans (mean) through the cheek region of the kernel shown in b). b)Intensity profile showing distribution of autofluorescence in an unstained oat kernel.

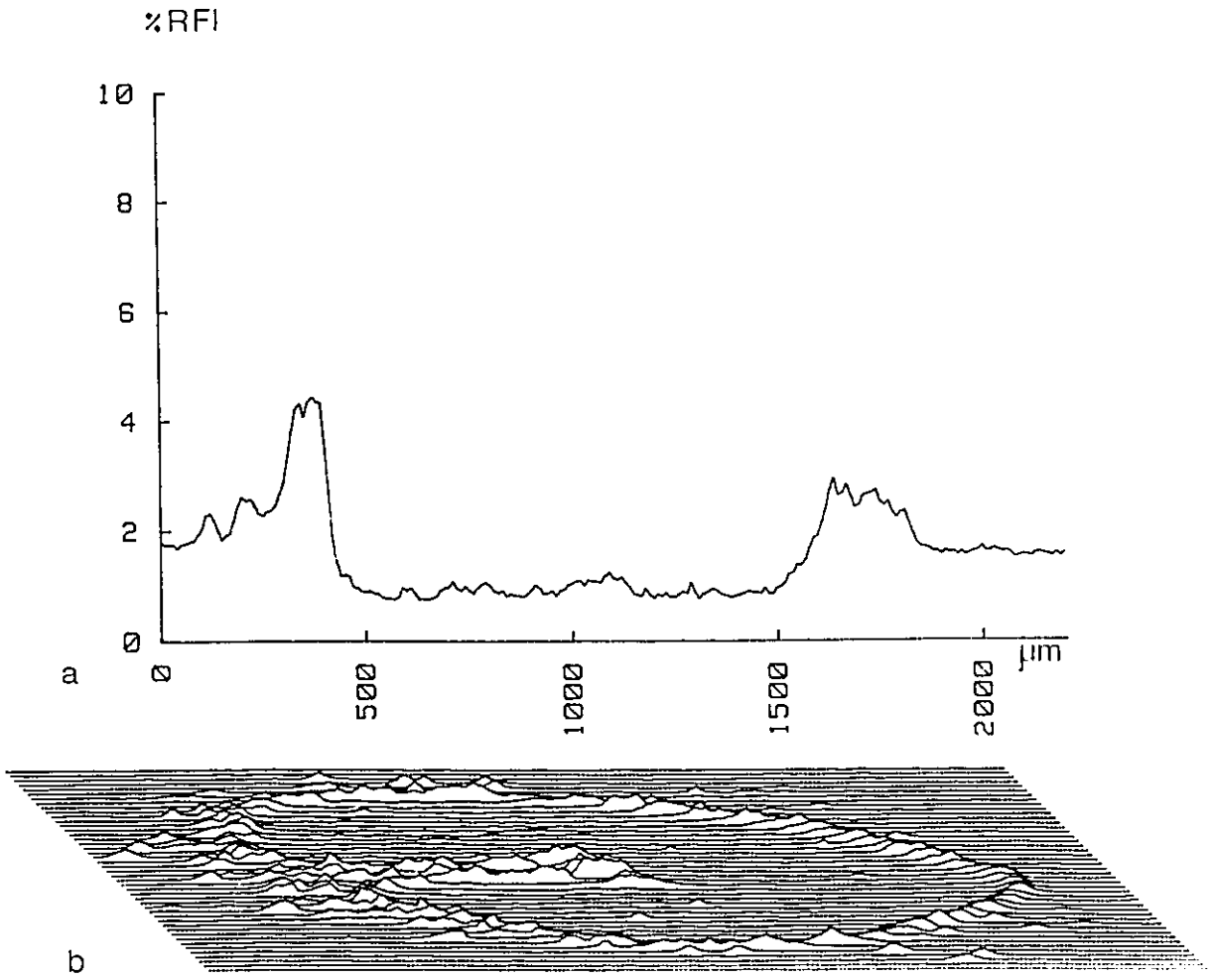
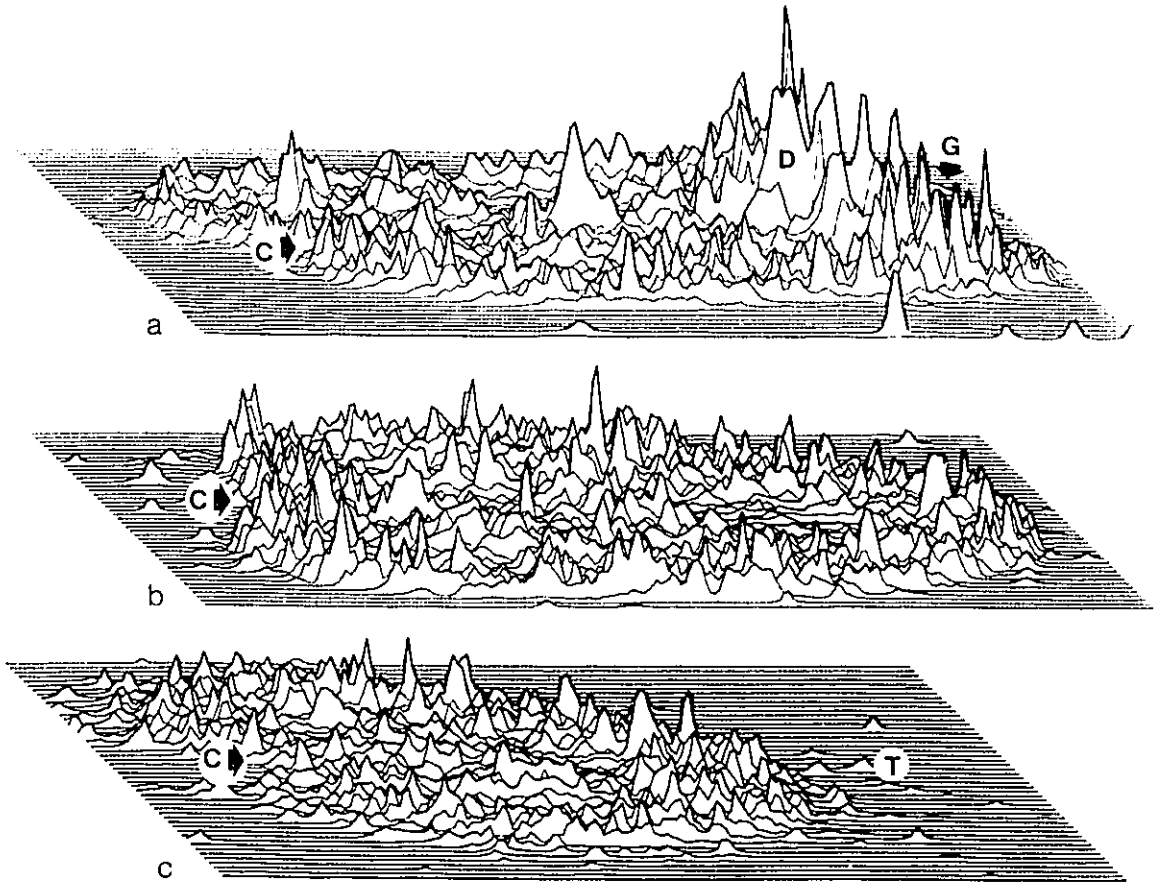

%RFI
10
8
6
4
2
0
0
500
1000
1500
2000
µm
a
b

Figure 12. Distribution of ß-glucan in a kernel of Woodstock oats, detected by scanning the relative fluorescence intensity of bound Calcofluor. a) Intensity profile of relative fluorescence intensity at the proximal end of the kernel (containing the embryo); b) Intensity profile of relative fluorescence intensity in the central region of the kernel; c) Intensity profile of relative fluorescence intensity at the distal end of the kernel.
G, germ; D, depleted layer; C, crease; T, trichomes.


D
G
C
a
C
b
C
T
c

The pattern of distribution is changed in the cultivar Marion (Fig. 13) which had a ß-glucan content of 6.35% for this particular sample. The greatest difference was seen in the central region of the kernel (Fig. 13b) where the RFI was high throughout the section and no clear sub-aleurone concentration of ß-glucan was observed. The concentration of ß-glucan was slightly lower towards the dorsal side of the kernel. A relatively even distribution throughout the kernel was also observed in the distal portion of the kernel (Fig. 13c). When thin sections of the central region of both Marion and OA516-2 were examined microscopically (Fig. 10c,d), the differences in ß-glucan content in the interior of the starchy endosperm appeared to reflect differences in cell size, rather than wall thickness. In OA516-2, the cells in the starchy endosperm were quite large (Fig. 10c), while in Marion the cells were much smaller (Fig. 10d), so that there appeared to be a higher proportion of cell wall per unit area in Marion than in OA516-2, possibly explaining the higher whole-grain content of ß-glucan in Marion. It is not known at this time whether the apparent differences in cell size in the starchy endosperm are found in other cultivars with different levels of ß-glucan. In the proximal region (Fig. 13a), the distribution of ß-glucan was similar to that seen in OA516-2 and in Woodstock, although the relative amounts were higher.

When the mean %RFI of central scans from 5 cultivars of oats (Fig. 14) was compared with ß-glucan content (determined enzymatically), a correlation coefficient of 0.97 was obtained (Fig. 15). The central region of the kernel was chosen for comparison because it constituted the largest proportion of the seed (roughly 50%), and might, therefore, provide the most accurate reflection of the total ß-glucan content of the kernel. The pattern of higher concentration of ß-glucan around the periphery of the kernel and lower amounts in the interior was consistent in all three of the lower ß-glucan cultivars

Figure 13. Distribution of ß-glucan in a kernel of Marion oats, detected by scanning the relative fluorescence intensity of bound Calcofluor. a) Intensity profile of relative fluorescence intensity at the proximal end of the kernel (containing the embryo); b) Intensity profile of relative fluorescence intensity in the central region of the kernel; c) Intensity profile of relative fluorescence intensity at the distal end of the kernel.
G, germ; D, depleted layer; C, crease; T, trichomes.

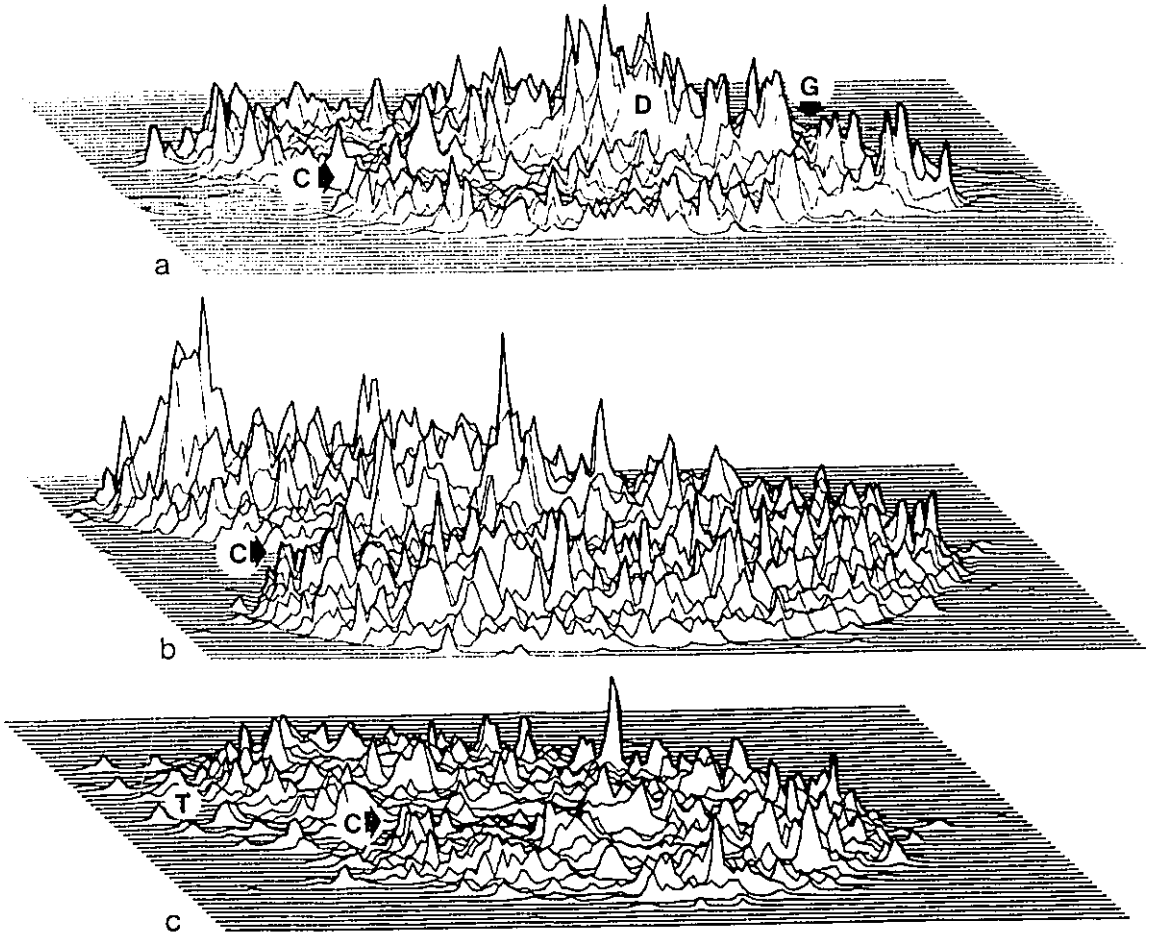

G
D
C
a
C
b
T
C
c

Figure 14. Comparison of ß-glucan distribution in the central region of 5 cultivars of oats: Intensity profiles showing distribution of bound Calcofluor. a) Donald (3.68% ß-glucan); b) OA516-2 (3.98% ß-glucan); c) Tibor (4.56% ß-glucan); d) Woodstock (5.07% ß-glucan); e) Marion (6.35% ß-glucan).

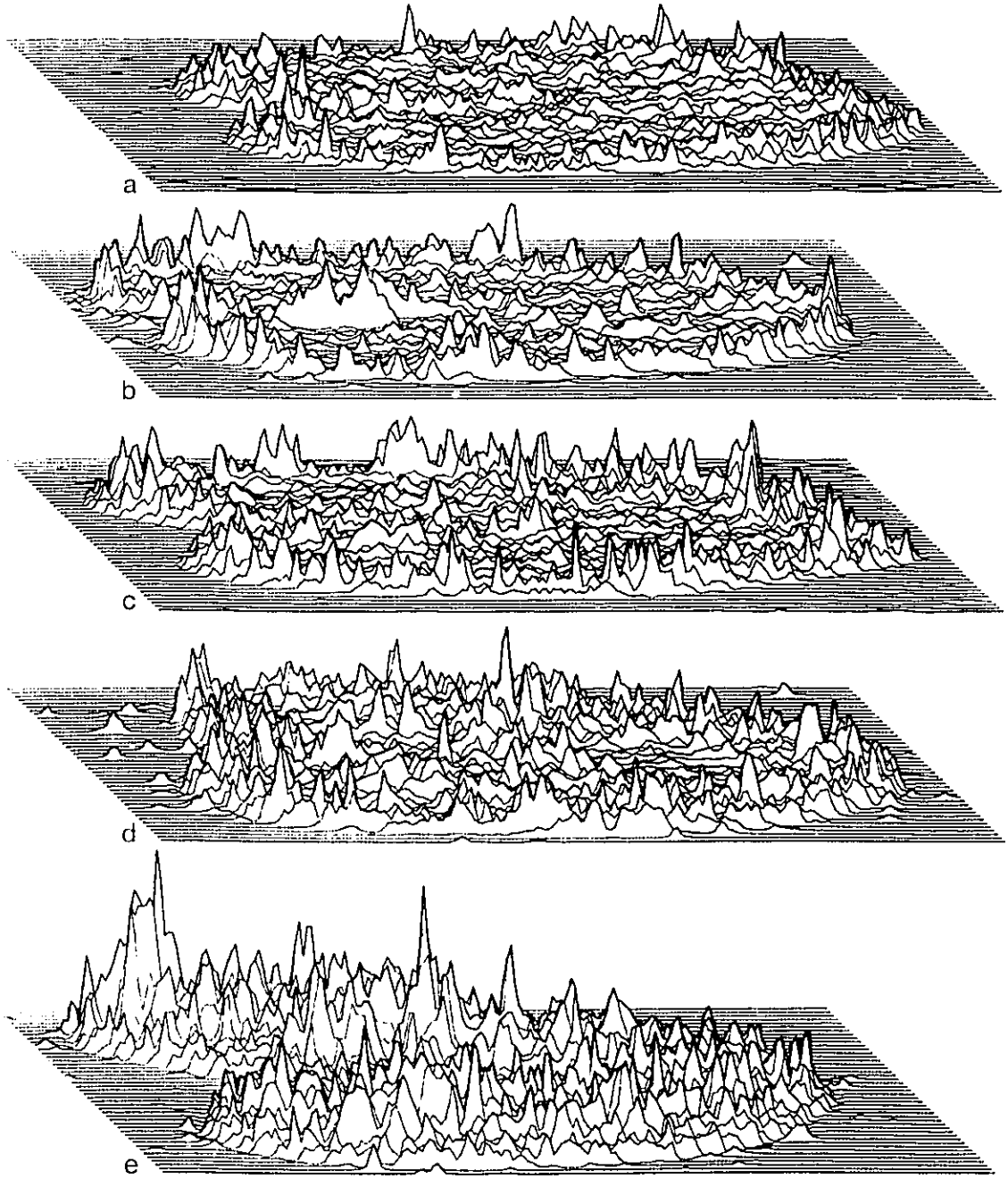
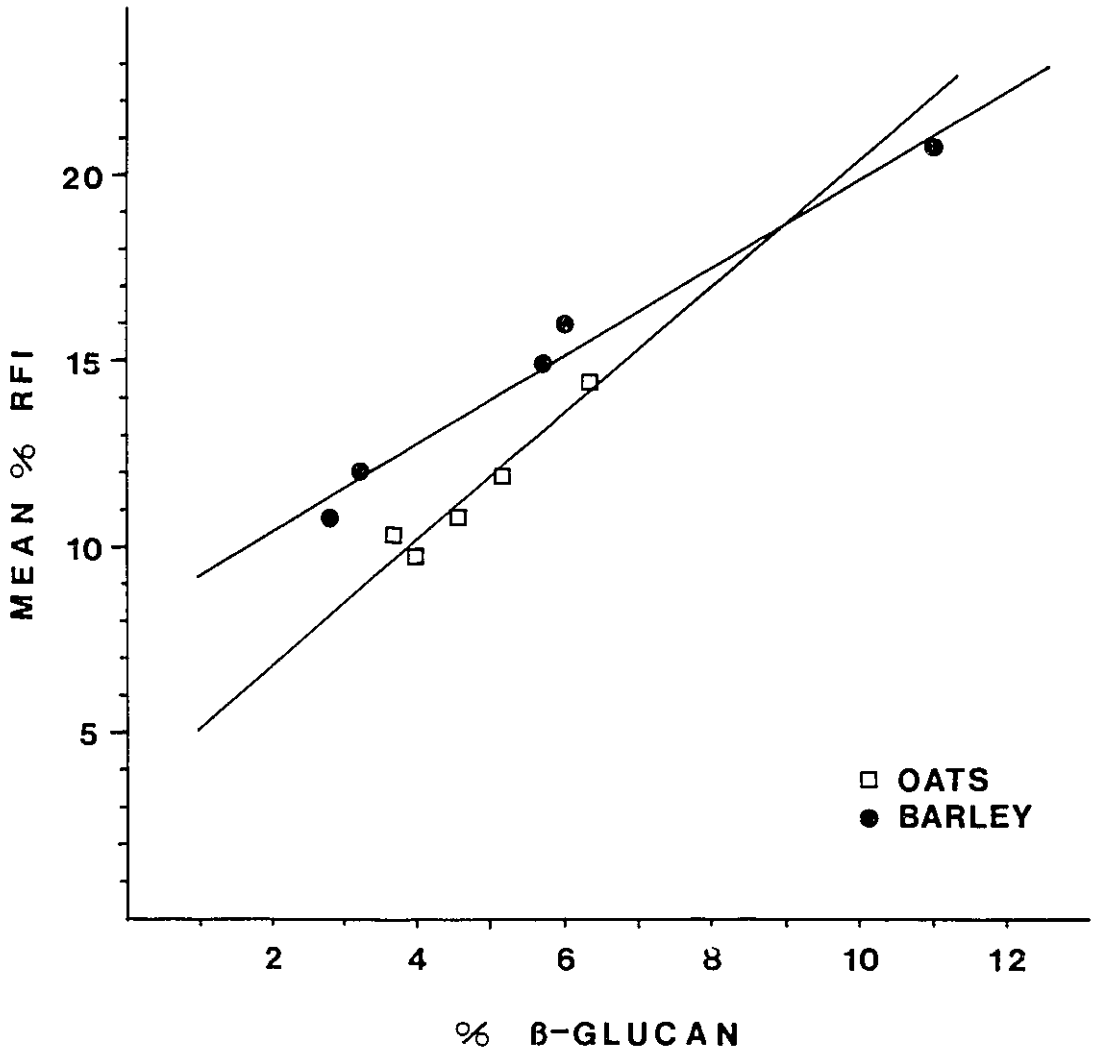

a
b
c
d
e

Figure 15. Comparison of mean relative fluorescence intensity (RFI) with % ß-glucan determined enzymatically in 5 cultivars of oats (open squares, r=0.97), and in 5 cultivars of barley (solid circles, r=0.99).


MEAN % RFI
20
15
10
5
2
4
6
8
10
12
% β-GLUCAN
OATS
BARLEY

(Donald, OA516-2, and Tibor; Fig. 14). The pattern became less pronounced as the ß-glucan content increased (Woodstock) and was lost in the cultivar with the highest ß-glucan content (Marion).

The high correlation observed between ß-glucan content and %RFI suggests that the microspectrofluorometer could be used for screening purposes. While the equipment required is too complex and expensive to be considered for routine purposes, the possibility of selection of single seeds (e.g. for breeding purposes) is very attractive. The distal portion of kernels of interest could be removed and scanned, and the remainder of the seed, with its embryo intact, would then still be viable.

Although the data presented in Figure 14 is by no means comprehensive, representing as it does only 5 cultivars of oats, a trend is apparent where the high sub-aleurone concentration of β-glucan becomes less distinct as the total β-glucan content of the cultivars increased. The lack of a distinct sub-aleurone concentration of β-glucan in the kernel may reflect differences in cell wall thickness in this area for some cultivars, as noted by Fulcher (1986), or it may be that a higher concentration of β-glucan in the central endosperm (for example, as in Marion) obscures any local high sub-aleurone concentration in the cultivars with higher β-glucan contents when examined using microspectrofluorometry.

The large difference in whole grain ß-glucan content observed between Marion and OA516-2 (the cultivars with the highest and lowest ß-glucan contents, respectively) was not reflected in isolated endosperm cell walls (See Section 4). Rather, microscopic evidence suggested that the differences in whole-grain β-glucan contents could be attributed, in part, to differences in the central endosperm as well as in the sub-aleurone layer, and also to the size and shape of the cells themselves (Fig. 10c,d).

Although the distribution of ß-glucan varied among the oats studied, a consistent feature in each of the cultivars examined was a large deposition of β-glucan in the portion of the starchy endosperm immediately adjacent to the embryo (Figs. 9a, 10a, 12a, 13a). Fluorescence micrographs of Calcofluor-stained sections of this area were similar for both high and low β-glucan cultivars.

The distribution of β-glucan through the central and distal regions of the kernel was much like the distribution in the ventral portion of the embryo region of the kernel for each cultivar, although this distribution differed between cultivars (Figs 9, 12, 13 and 15). In some cultivars, particularly those with low β-glucan contents, microspectrofluorometric scans indicated that there was a higher deposition of β-glucan around the periphery of the kernel. Microscopic examination of Calcofluor-stained sections of kernels of both Marion and OA516-2 showed the presence of thick, highly fluorescent cell walls in the sub-aleurone (Fig. 10b). Thickened cell walls were previously observed in the sub-aleurone layer of some cultivars of oats (Fulcher 1986).

Thickened cell walls in the sub-aleurone layers of oats have particular relevance in the production of oat bran, which is currently in increased demand due to clinical benefits which have been reported to accompany inclusion of oat soluble dietary fibre in the diet. Wood *et al* (1991b) reported an average enrichment factor of 1.4 in ß-glucan content between whole groats and brans from a selection of oats. Significant changes in ranking, however, were observed in the ß-glucan contents of kernels and brans. The differences in distribution observed in the present study could provide some explanation for the observations of Wood *et al* (1991b). In addition, it has become obvious that scanning microspectrofluorometry presents a valuable tool for selection of appropriate cultivars of oats for production of high ß-glucan brans.

The physicochemical properties of ß-glucan, particularly its high water-holding capacity, coupled with the distribution patterns observed in some cultivars of oats, suggest possible roles for this polysaccharide in the oat kernel. Perhaps the most obvious possibility is one of drought protection for the germinating embryo. When seeds are imbibed, prior to germination, the ß-glucan in the cell walls would absorb water and become a gel-like matrix. If the germinating seed were then subjected to drought stress, the hydrated ß-glucan around the outside of the seed and next to the growing embryo would prevent or delay desiccation, allowing growth to continue until the stress was relieved.

Alternatively, the hydrated walls could serve a transport function in germinating seeds. The deposition of ß-glucan in oat kernels is greatest in the areas immediately adjacent to the scutellar epithelium (in the embryo, see Fig. 1) and the aleurone layer. These tissues are the sites of synthesis and secretion of enzymes responsible for the breakdown of the reserves in the endosperm of cereal grains during germination (Brown and Morris 1890; Dure 1960a,b; Gibbons 1979; Okamoto and Akazawa 1979; Gibbons 1980; MacGregor *et al* 1984; Fincher 1989). The hydrated ß-glucan matrix of the thick cell walls in the endosperm adjoining these tissues could be routes of passive transport of enzymes into the starchy endosperm during germination, and possibly a transport route within the kernel for hydrolysis products and other solutes as well.

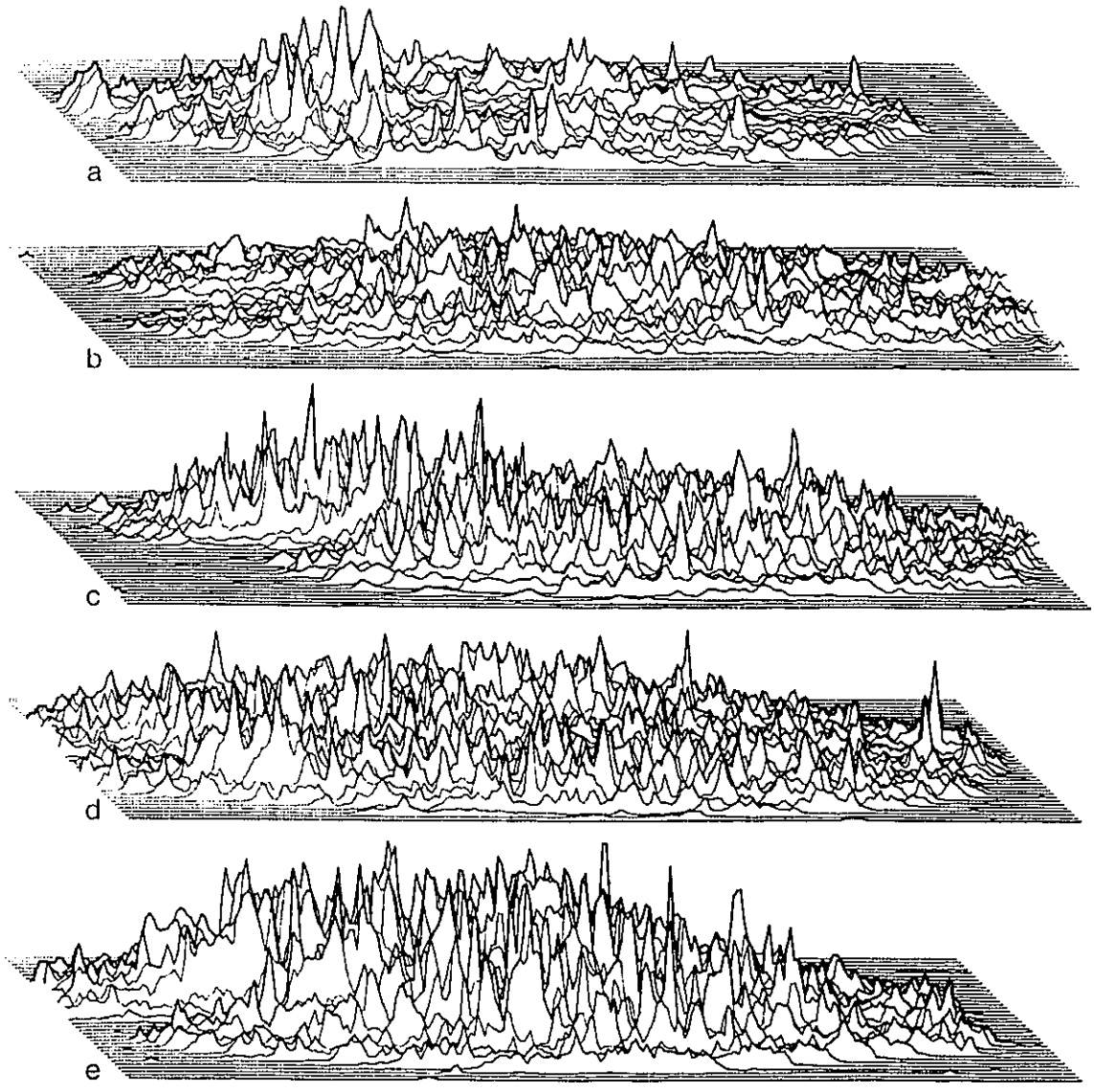
Distribution of ß-glucan in barley: As mentioned previously, barley also has high levels of ß-glucan in the kernel. A selection of barley cultivars with a broad range of ß-glucan contents was examined to determine whether the distribution patterns observed in oats were also present in barley. The distribution of ß-glucan in barley kernels (Fig 16) differed

from that in the oats examined. The peripheral area in kernels of all 5 of the barleys examined appeared to have lower concentrations of ß-glucan than the central endosperm. The dorsal and exterior cheek areas also appeared to have a lower concentration of ß-glucan than the central portion of the kernels in cross section (Fig. 16).

The range of ß-glucan content in the five barley cultivars was much wider than that determined for domestic oat cultivars. The barley samples contained one mutant with a very low ß-glucan content (M737, 2.8% ß-glucan) and one cultivar with an extremely high ß-glucan content (Arizona Hull-less, 11.0% ß-glucan). Differences in ß-glucan content have been related to differences in cell wall thickness in barley (Aastrup 1983). The low β-glucan cultivar shown in Fig. 16a, M-737, was produced from the higher β-glucan cultivar Minerva (Fig 16d) by mutation with sodium azide, and has cell walls approximately half as thick as those in the parent variety (Aastrup 1983).

As was observed for oats, the mean %RFI correlated strongly with β-glucan content (determined enzymatically) in barley (r=0.99, Fig. 15), although the slope of the lines for barley and oats were different. Four of the barley cultivars used in this preliminary study were the generous gift of Dr. S. Aastrup of the Carlsberg Research Laboratory in Copenhagen. The ß-glucan values used in the above correlation were kindly supplied by Dr. Aastrup, and because the samples obtained were quite small, the enzymatic analyses were not repeated in this study. As the correlation obtained was based on data from only 5 cultivars for each grain, it would be premature to draw conclusions from the differences observed. Nevertheless, it is interesting to speculate that the difference in regression lines might reflect differences in the structure of the β-glucans found in oats and barley, some of which have been documented (Wood *et al* 1991a). Alternatively, if the one barley sample with a very high β-glucan content (Arizona

Figure 16. Comparison of ß-glucan distribution in the central region of 5 cultivars of barley: Intensity profiles showing distribution of bound Calcofluor. a) M-737 (2.8% ß-glucan); b) Chalky Glen (3.2% ß-glucan); c) Leger (5.7% ß-glucan); d) Minerva (6.0% ß-glucan); e) Arizona Hull-less (11.0% ß-glucan).


a
b
c
d
e

Hull-less, 11.0% ß-glucan) was removed, the regression lines for the oat and barley data would be very nearly parallel. More data points would be required to determine whether the remaining distance between the two regression lines is significant, or merely an artefact of the small sample size.

While the different distribution pattern for the ß-glucans in barley as compared to oats could be considered a refutation of the role(s) tentatively advanced for ß-glucan in oats, barley differs from oats in other characteristics as well. The aleurone layer of oats is generally a single layer thick; in barley this tissue is three to four cell layers thick. Aleurone cell walls, which, like the endosperm cell walls contain some ß-glucan, are generally much thicker than those of the central endosperm in both of these cereals. The multiple layers of thick-walled aleurone cells could afford the same level of protection as the thick-walled sub-aleurone layer in oats. In addition, the thinner cell walls in the starchy endosperm in the sub-aleurone layer would not be as impenetrable as similar areas in oats. The potentially increased synthetic capacity of a multi-cellular aleurone layer, coupled with the more easily degraded and/or penetrated sub-aleurone walls could add up to a system that is roughly equivalent physiologically to that suggested for oats.

Distribution of ß-glucan in wheat: Wheat contains much less ß-glucan than either oats or barley, with levels seldom as high as 1% on a dry weight basis (Beresford and Stone 1983, Fincher and Stone 1986, Henry 1987). The distribution of β-glucan in kernels of wheat was similar to that observed in low β-glucan cultivars of oats, with the majority of the bound Calcofluor being observed around the periphery of the seed (Fig. 17a) and in the depleted layer (not shown). The central endosperm contained much lower β-glucan than was observed in the endosperm of even the lowest oat cultivar measured, with RFI

Figure 17. Distribution of ß-glucan in the central region of a kernel of wheat (cultivar Houser). a) Profile showing the average relative fluorescence intensity (y-axis) of 5 scans through the cheek region of the kernel shown in b); b) Intensity profile showing the distribution of Calcofluor bound to ß-glucan in a cross section through the central region.

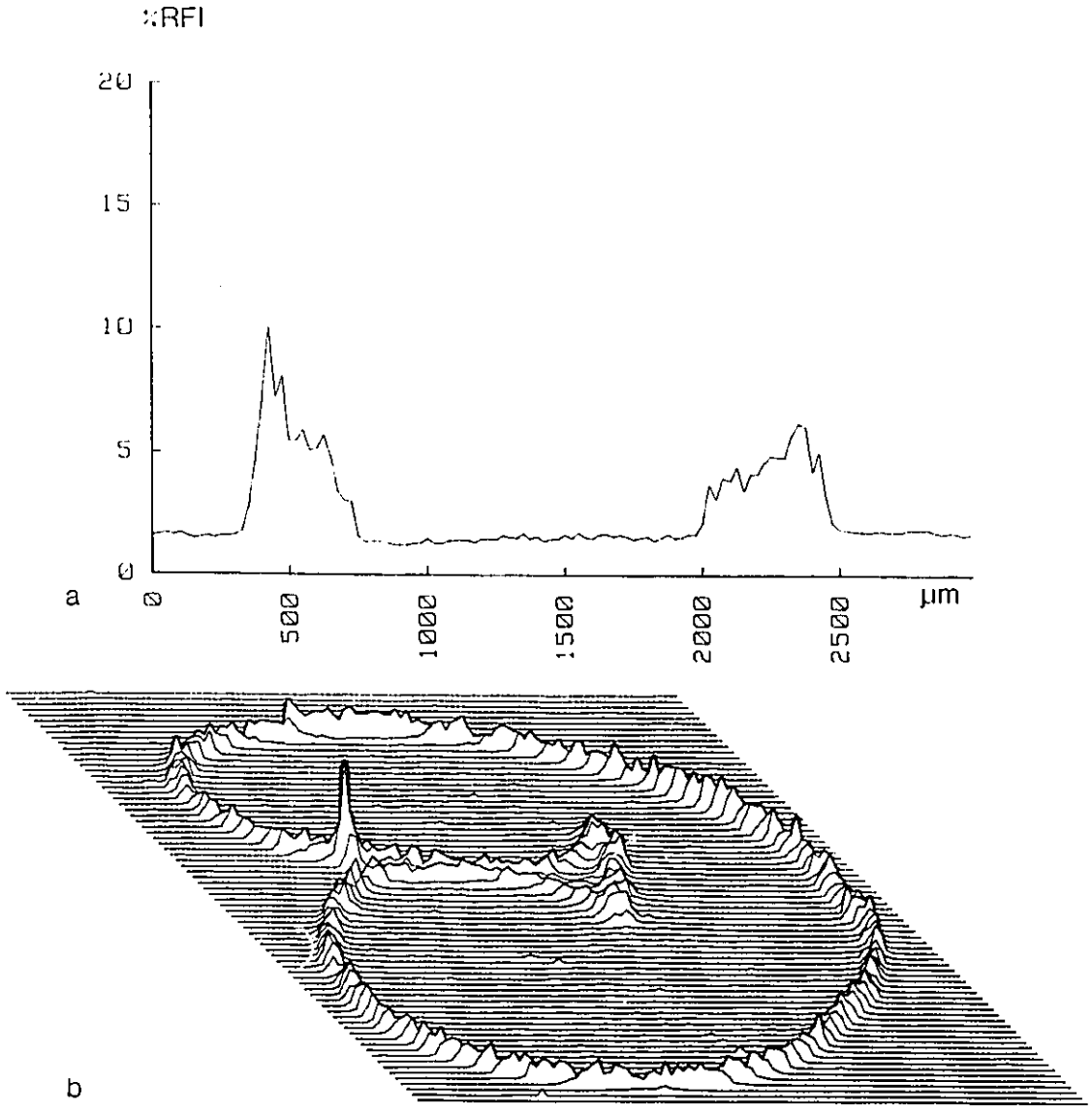

%RFI
20
15
10
5
0
a
0
500
1000
1500
2000
2500
µm
b

Table 15. ß-glucan content of milling fractions of wheat. Whole kernels were ground in a Udy grinder to produce whole grist, or milled in a flour mill to produce fractions equivalent to commercial flour (milled flour) and bran. ß-glucan was determined on duplicate samples of ambient moisture by FIA, and values converted to represent % on a dry weight basis.

Fraction	ß-Glucan (% dry wt.)
Whole grist	0.5
Milled flour	0.2
Bran	1.1

values well below the 5% low threshold which was used in these experiments (Fig. 17a). Although only a few wheats have been studied at this time, this pattern was consistently observed in all of the samples examined, and was confirmed by milling experiments. Whole grain samples of the wheat cultivar shown in Fig. 17 (Houser) contained 0.5% β-glucan (Table 15). This value is within the range previously reported for a wide selection of commercial and primitive wheats (Beresford and Stone 1983; Fincher and Stone 1986; Henry 1987). After milling, the bran fraction (which consists of pericarp and testa, aleurone, and most of the sub-aleurone layer as well) contained approximately double the amount of β-glucan that was determined in ground whole grain samples. The milled flour, which contains almost exclusively material from the starchy endosperm, contained only 0.2% β-glucan. Henry (1987) also noted a lower amount of β-glucan in wheat endosperm than in whole grain.

The distribution pattern observed in the wheat samples included in this study lends itself to the same (admittedly speculative) interpretation advanced for oats: protection, and/or a passive transport function in the germinating seed.

4. ISOLATION AND PRELIMINARY CHARACTERIZATION OF OAT ENDOSPERM CELL WALLS

4.1 Introduction

Microscopic evidence has shown that the endosperm cell wall is the main repository of ß-glucan in oats (Wood and Fulcher 1978; Wood and Fulcher 1983; Fulcher 1986; Section 3, Fig. 10). In order to gain a better understanding of both the biological and clinical roles of oat ß-glucan, a study of oat endosperm cell walls was indicated. To date, however, only mixed preparations of cell walls from oats, containing cell walls from both bran and endosperm, have been isolated (Selvendran and DuPont 1980; Aspinall and Carpenter 1984). These studies have yielded conflicting data on the nature and proportions of the cell wall constituents of oat grains. In addition, these cell walls were isolated in aqeous media, a procedure which greatly increased the probability of losses of water-soluble polymers from the cell walls.

The present study analyzed pure oat endosperm cell walls. The method developed by Mares and Stone (1973a) for isolation of wheat endosperm cell walls was selected as a starting point. This method (which is performed in 70% ethanol to avoid loss of water-soluble components) required some modification, however, to solve the problem of coprecipitation of cytoplasmic compounds (Selvendran and DuPont 1980), as well as new problems arising from the chemical and morphological differences between wheat and oats, which were encountered as attempts at cell wall isolation progressed. Only after these problems were solved was it possible to isolate sufficient quantities of pure endosperm cell walls for analysis.

Endosperm cell walls were isolated from two cultivars of oats: one with a high content of ß-glucan (Marion) and one with a low content of ß-glucan (OA516-2). Initial experiments to characterize the cell wall components were performed on unfractionated cell walls. These analyses included ß-glucan analysis (using the modified enzymatic method described in Section 3); acid hydrolysis of cell walls and identification and quantitation of the resulting monosaccharides; alkaline extraction for characterization of cell wall phenolics; and cell wall hydrolysis for analysis of the amino acid profile. The cell walls were then fractionated by sequential hot water extraction, lichenase digestion and xylanase digestion. The final residue after xylanase digestion was hydrolyzed in acid, and the monosaccharide composition determined. This fractionation protocol was chosen to minimize the degradation and non-specific bond cleavage that can occur in the solvents used in the traditional chemical fractionation of plant cell walls.

The studies reported in this section provide an improved understanding of the structure and biochemistry of the oat endosperm cell wall. Such knowledge helps to define our concept of the role of ß-glucan in the seed, and can also be applied in nutritional studies of oats and oat products.

4.2 Experimental Procedures

4.2.1 Cell wall isolation

For isolation of plant cell walls, a modification of the method of Mares and Stone (1973a) was used. Oats were first dehulled, and the resulting groats tumbled for 48 hours in an elbow blender lined with coarse sandpaper to polish off trichomes and a substantial amount of the pericarp and testa. The polished groats were then washed with 70% ethanol to remove any loosely adhering material, and air dried before grinding in a UDY

cyclone grinder (UD Corporation, Fort Collins, Colorado) to pass a 1 mm screen. The resulting flour was defatted using petroleum ether, and air dried. The defatted flour was extracted for 4 hours in 70% ethanol and wet sieving repeated. Microscopic examination showed that the material passing through the screen was largely intracellular starch and protein. The material retained by the screen was extracted further in 70% ethanol for one hour, and wet sieved again through a 73 μm screen. The material retained by the screen was made into a thick slurry with 70% ethanol and homogenized using a Polytron (Sybron-Brinkman) to break up the cells and free the cell walls from the cell contents. Following homogenization, the slurry was again wet seived through a 48 μm and a 30 μm screen. The homogenization followed by wet sieving was repeated until a significant amount of cell wall material was released. This was determined by the presence of a "fluffy" layer on top of the sediment. The entire sediment was then suspended in 70% ethanol in a large (2 L) beaker, and allowed to settle. The heavier fragments, containing clumps of cells and cell contents (sludge), were allowed to settle out, and the cell walls were harvested by aspiration. The sedimentation/aspiration step was repeated several times to maximize the yield of cell walls.

The crude cell wall preparation was then washed with 1% sodium dodecyl sulfate (SDS) in 70% ethanol to facilitate removal of small starch granules and cytoplasmic protein adhering to the cell walls. The contaminating bran (aleurone, pericarp and seed coat; see Fig. 1) cell walls were removed by the addition of AG 1-X2 anion exchange resin (BioRad Laboratories, Richmond, California) in the formate form suspended in 70% ethanol. The loose complexes formed by the bran cell walls and the anion exchange beads were allowed to settle, and the remaining endosperm cell walls were harvested by

aspiration. A schematic diagram outlining the isolation procedure is presented in Figure 18.

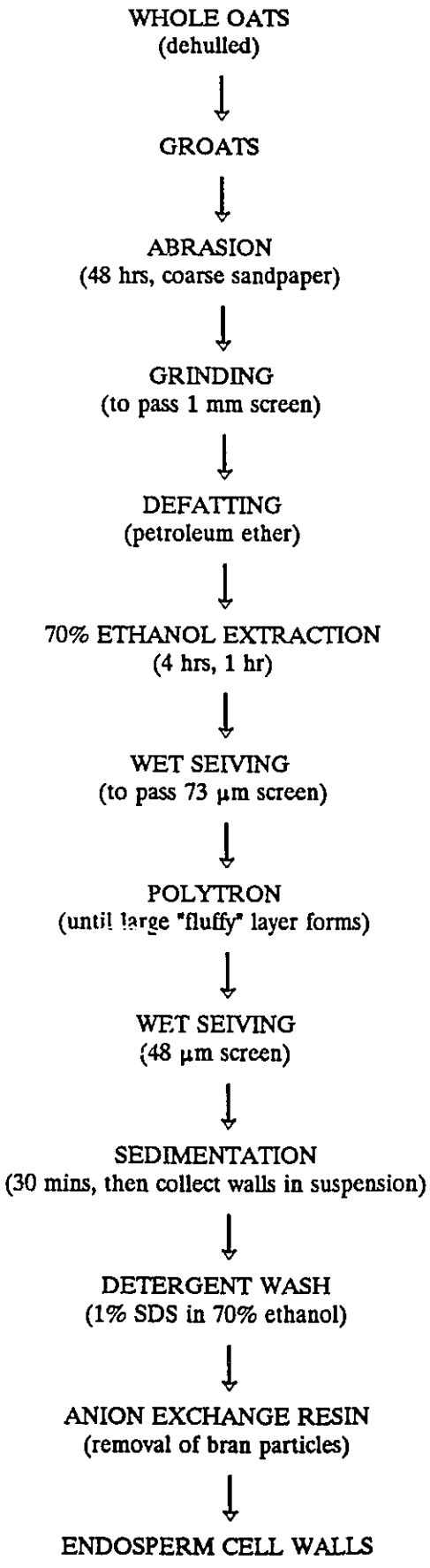
The purified endosperm cell walls, which contained about 5% residual ion exchange resin contamination (estimated microscopically), were either stored in 70% ethanol, or solvent dried (95% ethanol, 100% ethanol, isopropanol) and then air dried before analysis.

4.2.2 Preliminary characterization of the oat endosperm cell wall

4.2.2.1 Monosaccharide analysis of oat endosperm cell walls

GC analysis of monosaccharides: Cell wall samples (3-4 mg) were hydrolyzed in 2.0N sulfuric acid at 100°C for 3 hours. To estimate the cellulose component, a parallel sample of each cell wall preparation was subjected to a primary hydrolysis in 72% sulfuric acid (w/w) at 4°C for one hour, then diluted to 2.0N withdistilled water, and hydrolyzed at 100°C for 2 hours. The samples were cooled, neutralized by the addition of barium carbonate and filtered through pulp pads. After addition of sodium borohydride (15-20 mg) to reduce monosaccharides, the samples were allowed to stand at room temperature overnight. Excess borohydride was destroyed by the addition of glacial acetic acid, and sodium ions were removed by Rexyn 101. The samples were filtered and concentrated to dryness, and borate was removed by repeated addition and evaporation of anhydrous methanol. The reduced monosaccharides were then acetylated by addition of pyridine and acetic anhydride, and incubated at 100°C for one hour under anhydrous conditions. Complete acetylation was assured by allowing the samples to stand at room temperature overnight after the 100° incubation. A few drops of water were added to the samples, which were then evaporated to dryness. This was repeated 3 times to ensure

Figure 18. Flow chart showing the sequence of steps required for isolation of cat endosperm cell walls.


WHOLE OATS
(dehulled)
GROATS
ABRASION
(48 hrs, coarse sandpaper)
GRINDING
(to pass 1 mm screen)
DEFATTING
(petroleum ether)
70% ETHANOL EXTRACTION
(4 hrs, 1 hr)
WET SEIVING
(to pass 73 µm screen)
POLYTRON
(until large "fluffy" layer forms)
WET SEIVING
(48 µm screen)
SEDIMENTATION
(30 mins, then collect walls in suspension)
DETERGENT WASH
(1% SDS in 70% ethanol)
ANION EXCHANGE RESIN
(removal of bran particles)
ENDOSPERM CELL WALLS

complete removal of pyridine and acetic anhydride from the sample. The reduced, acetylated monosaccharides were taken up in 1 mL of chloroform, and stored in the fridge until analysis by gas chromatography.

Gas chromatography (GC) analysis was performed with a Varian Vista 6000 Gas Chromatograph, equipped with a flame ionization detector: DB-225 fused silica capillary column (0.25 μm film, 30 m x 0.32 mm (i.d.), cold column injection); initial temperature 50°C with a hold time of 2.5 min, increased to 250°C at a rate of 5°C/min; helium flow rate 1.5 mL/min; nitrogen makeup gas (30 mL/min); injector temperature 180°C/min from 30 to 220°C. Peak areas were evaluated with a Vista 402 data system.

HPLC analysis of monosaccharides: For analysis of the monosaccharide profile of the endosperm cell walls by HPLC, samples were hydrolyzed with H_2SO_4 (2.0N and 72%), neutralized with barium carbonate, and filtered through pulp pads as above. Standard mixtures of sugars containing glucose, xylose, arabinose, galactose and mannose were hydrolyzed under the same conditions. The samples were then flash evaporated and made up to 1 mL with distilled water. Samples were analyzed using a Varian Series RI-3 Refractive Index detector equipped with a Varian 2510 HPLC pump. Monosaccharides were separated on an Aminex HPX-87P (BioRad) column, 300 x 7.8 mm. The flow rate was 0.6 mL/min, at a temperature of 85°C and the mobile phase was water. All samples and standards were filtered through 0.45 μm filters and injected into a 10 μL fixed volume loop. Samples containing very small quantities of sugars were analyzed without neutralization using a Dionex HPLC, with pulsed amperometric detection (as for analysis of tri- and tetrasaccharides, see Section 3). Samples were hydrolyzed as above, diluted

to reduce acid strength, and filtered through a 0.45 μm filter before injection. The column was regenerated after each sample by a 30 minute run with dilute sodium hydroxide.

4.2.2.2 Methylation of oat endosperm cell walls

Cell walls of small particle size (retained on a 30 μm screen) were methylated by the procedure of Hakamori (1964). Dried cell walls in anhydrous DMSO were methylated by 3 successive additions of methylsulfinyl carbanion and methyl iodide, added dropwise with a syringe through a serum cap while flushing with N_2, over 24 hours to ensure complete methylation. After overnight dialysis against running tap water, the product was recovered by continuous extraction with chloroform. Because the IR spectrum (recorded with a Beckman IR 4250 spectrophotometer) of the sample indicated the presence of underivatized hydroxyl, the methylation procedure was repeated. The final product ($[\alpha]_D^{25}$ -4.8°) showed no IR absorption for hydroxyl.

The methylated sample was refluxed in methanolic HCl (24 hrs), neutralized by addition of Ag_2CO_3, and hydrolyzed (1.0N H_2SO_4, 100°C, 30 hrs; neutralization with $BaCO_3$). A portion of this mixture was reduced and acetylated (as above), and the products examined by GC. A sample was also submitted at the Plant Research Centre, Agriculture Canada, for GC-Mass Spectrometry analysis.

4.2.2.3 Amino acid analysis of endosperm cell walls

Cell wall samples were hydrolyzed in 6.0N HCl, at 110°C under N_2 for 23 hours. Corrections for losses over this period were not made. The samples were then freeze-dried to remove the acid, and reconstituted in sodium citrate buffer (0.2 M, pH 2.0). Sugars were removed by passing the sample through a Dowex column (H^+ form) and

washing through with distilled water; amino acids were subsequently eluted with pyridine. The hydrolysates were freeze-dried again, reconstituted in citrate buffer (as above), and spun down to remove any particulate material. Amino acid analysis was performed using a Technicon TSM analyzer.

4.2.2.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of endosperm cell wall extracts

Purified endosperm cell walls were extracted in boiling Phast buffer (10 mM Tris, pH 6.8; 1 mM EDTA; 5% SDS; 5% ß-mercaptoethanol; 10% glycerol; 1% bromphenol blue) for 30 minutes, then centrifuged, and the extract (10 µL) loaded onto commercially prepared polyacrylamide Phast mini-gels (gradient 10-15%) for electrophoresis. The gels were run on the Phast system (Pharmacia, Sweden) before fixing and staining with Coomassie Blue, or silver stain. In some cases, walls were extracted overnight with 8 M urea (37°C, then 80°C for 1 hr), or digested with lichenase prior to the Phast buffer extraction.

4.2.2.5 Analysis of cell wall phenolics

Samples were extracted with 2.0N NaOH under N_2 at room temperature with shaking for 4 hours. At the end of the extraction the pH of the samples was adjusted to 2.0 ± 0.1 by the addition of 6.0N HCl. The samples were then centrifuged for 30 minutes at 2000 rpm. Pellets were washed twice with distilled water. The supernatants from the initial centrifugation and subsequent washings were extracted individually with ethyl acetate (2 x 50 mL) and then pooled for a final extraction with 50 mL of ethyl acetate. Sodium sulfate was added to the samples to absorb any water from the ethyl acetate.

After standing overnight, the samples were filtered, and evaporated to dryness. The dried extracts were taken up in 1.0 mL of ethyl acetate, dried again under N_2, and finally taken up in 1.0 mL of methanol.

Samples were analyzed using a Perkin-Elmer LSC 480 Diode Array Scan detector (at 280 nm) equipped with a Perkin-Elmer LC 250 pump. Phenolic acids were separated on a 250 x 4.6 mm C_{18} reverse phase column (Altex, Ultrasphere) with a Lichrosorb C_{18} pre-column. The mobile phase was an isocratic mixture of methanol and citrate buffer (5 mM, pH 5.4) (20:80), and the flow rate was 1.0 mL/min. All samples and standards were filtered through 0.45 µm Nylon 66 filters and injected into a 20 µL fixed volume loop.

4.2.2.6 Cell wall fractionation

Hot water extraction: Cell wall samples (80-100 mg) were first extracted exhaustively (until the extract was negative to Anthrone reagent) with distilled water at 65°C. The extract was collected by centrifugation (15 min, 10,000 x g), then freeze-dried and weighed before reconstituting with sodium phosphate buffer (20 mM, pH 6.5; 1 mL/mg extract), and redissolved by stirring overnight at 40°C. Lichenase (250 µL/mL buffer, 50 units/mL) was added, and the sample incubated with stirring for 4 hrs at 40°C. Each sample was then centrifuged (15 min, 10,000 x g) to remove insolubles generated during lichenase digestion, and the pellet washed. Both digest and pellet were freeze-dried. For separation of oligosaccharides, the freeze-dried digest was reconstituted in 5-6 mL of distilled water and filtered through a 0.45 µm filter before application on a BioGel P-2 gel permeation column.

The insoluble material generated during lichenase digestion was freeze-dried, pre-hydrolyzed in 72% H_2SO_4, diluted to 2.0N H_2SO_4 for hydrolysis at 100°C, and the monosaccharide composition determined using HPLC.

Lichenase digestion of hot water residue: Sodium phosphate buffer (20 mM, pH 6.5), and lichenase were added to the residue from the hot water extraction of the cell walls, and the samples incubated at 40°C with stirring for 4 hours. Samples were then centrifuged (15 min, 10,000 x g) and the pellet washed twice with distilled water. The digest and washings were pooled and freeze-dried before reconstitution and separation of oligosaccharides on BioGel P-2 as above.

Xylanase digestion of lichenase residue: The lichenase digested pellet was digested with xylanase in sodium acetate buffer (50 mM, pH 5.4) at 30°C with stirring overnight. The digest was collected by centrifugation (15 min, 10,000 x g), and the pellet washed twice with distilled water. The digest and washings were pooled and freeze-dried before reconstitution and separation of oligosaccharides on BioGel P-2 as described above. The final residue was also freeze-dried and weighed before acid hydrolysis (72% H_2SO_4) and HPLC analysis of monosaccharides.

4.2.2.7 Gel permeation chromatography

Oligosaccharides generated during cell wall fractionation were separated on two 90 x 2.6 cm BioGel P-2 (extra fine grade, minus 400 mesh, BioRad) columns connected in series. The mobile phase was distilled water containing 0.02% sodium azide to prevent microbial contamination. The water-jacketed columns (Pharmacia, Sweden) were run at

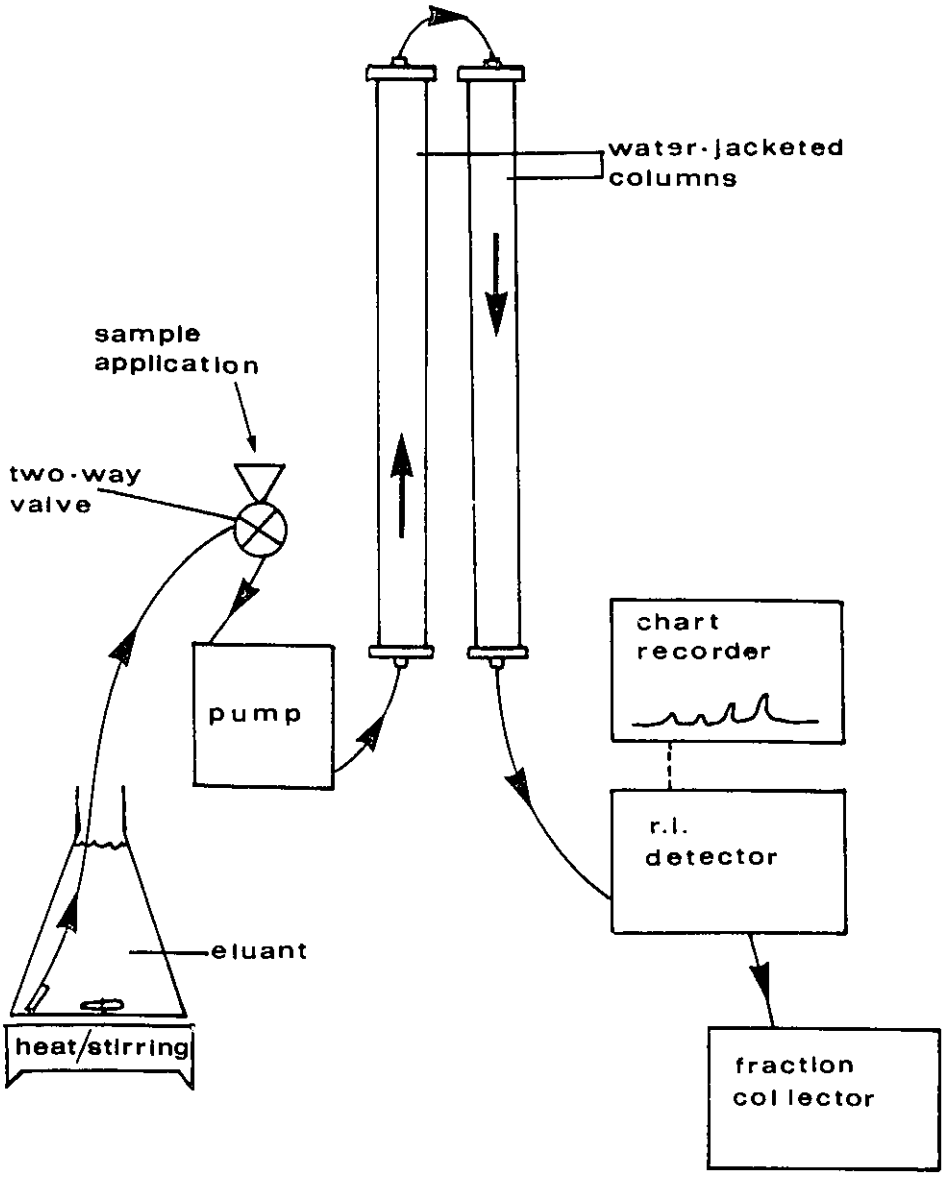
56°C. Samples were pumped through the columns at 24 mL/hr using an LKB 2120 Varioperpex II peristaltic pump (Pharmacia), and fractions (5 mL) collected with an LKB 2070 Ultrorac II fraction collector. Oligosaccharides were detected using an LKB 2142 refractive index detector installed between the second column and the fraction collector. A diagram illustrating the setup for gel permeation chromatography is presented in Figure 19. Peaks shown on chromatograms were pooled and freeze-dried before analysis.

The glucose content of fractions generated by lichenase digestion of the hot water extract and the hot water residue was determined by incubation of an appropriately diluted aliquot with ß-glucosidase in sodium acetate buffer followed by addition of glucose oxidase/peroxidase reagent (as for manual ß-glucan determination, see Section 3.2.2). The fractions generated by xylanase digestion were very small, and insufficient quantities were collected for linkage analysis by conventional methods. The separate fractions were, however, freeze-dried and hydrolyzed in 2.0N H_2SO_4, then diluted and applied directly to the HPLC with PAD to determine monosaccharide composition.

4.2.2.8 Growth and harvesting of *E. coli* clone PBX I for xylanase production

A culture of *Escherichia coli* strain HB101 with the recombinant plasmid pPBX I containing the xylanase gene from *Bacteroides succinogenes* was the generous gift of Dr. C.W. Forsberg (University of Guelph, Dept. of Microbiology; ref. Sipat *et al* 1987). An aliquot of overnight culture was used to inoculate fresh medium (1% tryptone, 0.5% yeast extract, 1% NaCl, 100 µg/mL ampicillin) first thing in the morning, and the culture incubated at 37°C with shaking until late log phase was reached ($O.D._{420}$~3). Xylanase was harvested following a modification of the protocol of Cornelis (Cornelis *et al* 1982).

Figure 19. Flow diagram for separation of oligosaccharides by gel permeation chromatography using BioGel P-2.


water-jacketed columns
sample application
two-way valve
pump
chart recorder
r.i. detector
eluant
heat/stirring
fraction collector

The culture was centrifuged at 6000 rpm for 15 minutes, and washed twice with Tris buffer (10 mM, pH 8.0, 1% NaCl). The cells were resuspended in the same volume of 25% sucrose containing 1mM EDTA, and incubated at room temperature with shaking for 10 minutes. After centrifugation at 7000 rpm for 10 minutes, the cells were quickly and vigorously resuspended in the same volume of ice water. The suspension was shaken for 10 minutes at 4°C, and centrifuged at 9000 rpm for 10 minutes. This procedure should effectively release protein located in the periplasmic space without disrupting the cell membrane. The supernatant containing the xylanase was carefully decanted from the pellet, and freeze-dried. For analysis of enzyme activity and use in cell wall fractionation, the freeze-dried material was reconstituted in acetate buffer (50 mM, pH 5.4). The protein concentration (BioRad protein assay kit, BioRad Laboratories, California) in the reconstituted preparation was 0.2 mg/ml.

Xylanase activity: Throughout the harvesting procedure, xylanase activity was monitored using the artificial substrate 4-O-methyl-D-glucurono-D-xylan-Remazol Brilliant Blue R (Remazol Brilliant Blue Xylan, Sigma), following the protocol of Biely *et al* (1985). More precise determinations of xylanase activity in the final reconstituted preparation were achieved by monitoring the release of reducing sugars from xylan (from oat spelts, Sigma) (John and Schmidt 1988). The specific activity of the preparation used was 578 μmoles xylose equivalents/min/mg protein.

4.2.2.9 Microscopic examination cell wall samples

Samples from the cell wall isolation procedure were examined using a Zeiss Universal Research Microscope (Carl Zeiss Canada Ltd.). The microscope was equipped with a

tungsten illuminator for examination of specimens by bright-field microscopy using transmitted light, and a III RS epi-illuminating condenser for fluorescence analysis. The fluorescence filter combination used for examination of autofluorescence and Calcofluor stained samples had a dichromatic beam splitter, and an exciter/barrier filter set with with maximum transmission at 365 nm/>420 nm. Photomicrographs were obtained using 35 mm Kodak Ektachrome 100 for bright-field images, and Ektachrome 400 for fluorescence.

Stains used for microscopy: For examination of cell walls and cell wall fractions, the following dyes were routinely used: Calcofluor White M2R New (American Cyanimid), 0.01% in 50% phosphate (10 mM, pH 8) buffered ethanol; Fast Green FCF (Fisher), 0.1% in acetate buffer (50 mM, pH 4.0); Toluidine Blue O (Sigma), 0.05% in acetate buffer (50 mM, pH 4.4); Congo Red (Aldrich), 0.01% in distilled water.

4.3 Results and Discussion

4.3.1 Isolation of the oat endosperm cell wall

To gain relevant information concerning cell walls, it is desirable to obtain walls from a single tissue, which can be separated from both intracellular components and contaminating cell walls by methods which do not alter either the composition or the structure of the cell walls of interest. The method developed by Mares and Stone (1973a) to isolate endosperm cell walls from wheat was chosen as a starting point for isolation of oat endosperm cell walls because the procedure was performed in 70% ethanol, thus preventing loss of water-soluble materials from the cell walls. Variations of this method have been used to isolate endosperm cell walls from barley as well (Fincher 1975, 1976; Palmer 1975; Ballance and Manners 1978; Ahluwalia and Ellis 1985; Ahluwalia and Fry

1986; Brunswick *et al* 1988). Because of differences in the shape and chemical composition of oats as compared to wheat or barley, however, a number of modifications were required. The oat kernel is long and narrow, and contains a much higher proportion of lipid than wheat or barley; conventional milling or pearling procedures (Mares and Stone 1973a; Fincher 1975; Ballance and Manners 1978; Ahluwalia and Ellis 1985; Ahluwalia and Fry 1986; Brunswick *et al* 1988) could not, therefore, be used to produce material that was enriched in endosperm tissue. In addition, the high lipid content of oats (Morrison 1978; Youngs 1986) makes the oat kernel much softer than wheat or barley, and the kernels tended to be crushed rather than broken open in the break rolls of a conventional flour mill. The oat material produced in the break and reduction rolls of the mill adhered together in clumps which would not pass through the sifting screens of the mill. Thus the milling of oats did not give the clean separation of endosperm and bran observed in the milling of wheat. Passage of the long, narrow oat kernels through a barley pearler produced seeds that had the ends removed, and the portion of the kernels remaining retained the majority of associated bran tissue.

The problem of obtaining an endosperm-enriched fraction from oats was finally resolved by tumbling groats for two days in an elbow blender lined with coarse sandpaper. In this procedure, much of the bran was abraded off the kernels, leaving groats that were enriched in endosperm tissue. Nevertheless, the amount of bran present was relatively more than that found in milled wheat or pearled barley. The portion of the groats removed by abrasion in the elbow blender contained mainly trichomes, and cell walls from the pericarp and testa, with smaller amounts of aleurone as well (Fig. 20a). Little if any endospermic material was observed in this fraction.

Figure 20. Micrographs of fractions obtained during isolation of oat endosperm cell walls. a) Abraded fraction, stained with Toluidine Blue O, containing primarily fragments of bran cell walls (large arrows) and trichomes (small arrows). b) Flour from abraded groats stained with Congo Red. Cell walls (arrows) are mixed with large quantities of the intracellular starch and protein matrix. c) Bran-enriched fraction, stained with Toluidine Blue O. Bran cell walls take up the dye (large arrows) while the endosperm cell walls remain colourless (small arrows). d) Crude endosperm fraction, stained with Fast Green. Endosperm cell walls (unstained) show areas contaminated with cytoplasmic protein (large arrows) and starch (small arrows). e) Removal of bran cell walls (purple) from the crude endosperm fraction by complexing with anion exchange beads (circular and unstained); preparation stained with Toluidine Blue O. f) Purified oat endosperm cell walls, stained with Fast Green.
a,b,c,d and f, magnification X16; e, magnification X6.3.

a
b
c
d
e

The abraded groats were ground and samples were defatted by extraction with petroleum ether because the high lipid content of oats caused the groat flour to clump together, hindering the removal of starch and protein from the sample. (Removal of lipid was not required for isolation of either wheat (Mares and Stone 1973a) or barley (Fincher 1975, Palmer 1975, Ballance and Manners 1978) endosperm cell walls). The ground, defatted oat sample contained intracellular starch and protein, as well as cell walls from the endosperm, and smaller amounts from the aleurone layer, pericarp and testa (Fig. 20b). The defatted material divided easily into very fine particles and samples could then be extracted in 70% ethanol to remove sugars and oligosaccharides as well as small peptides, but not water-soluble polysaccharides present in the wall. Although Mares and Stone (1973a) used a French Pressure cell to disrupt clumps of cells and help remove protein and starch granules adhering to the cell walls, later modifications to the method (Fincher 1975; Ballance and Manners 1978) used various types of homogenizers, which are more convenient to use and more easily controlled. The Polytron homogenizer also has an ultrasonic component, which assists in the removal of some protein and starch granules. Therefore, after ethanol extraction and wet sieving to remove loose starch and protein, the ground material was homogenized using a Polytron. This procedure effectively breaks up remaining clumps of cells, and helps dislodge starch and protein from the cell walls.

The homogenization process was repeated several times, with the sample wet sieved in between to separate the cell walls from the cytoplasmic contaminants. The resulting crude cell wall preparation containing both bran and endosperm cell walls was suspended in a 2.0L beaker and allowed to settle for 30 minutes. (The 5 minute settling recommended by Mares and Stone (1973a) was found to be insufficient.) The cell walls

that remained in suspension at the end of this time were collected by aspiration, and the settled cell walls were again re-suspended and allowed to settle. The process was repeated several times to ensure the maximum yield of endosperm cell walls. The settled portion was enriched with bran cell walls, although endosperm cell walls were present as well (Fig 20c).

A major problem with the isolation procedure of Mares and Stone (1973a) is the presence of residual starch granules and cytoplasmic protein adhering to the isolated walls (Selvendran and DuPont 1980). Both starch and protein were observed in the crude cell wall preparation (Fig. 20d). As the presence of these components, even in small amounts, would influence the determination of wall composition, a means was sought for removing them. α-Amylase has been reported to retain activity in 70% ethanol (Mares and Stone 1973a; Fincher 1975). Attempts to remove oat starch from endosperm cell wall preparations by this method, however, were unsuccessful. No amylolytic activity was detected in 70% ethanol using α-amylase from either human saliva or hog pancreas. The method proposed by Selvendran and DuPont (1980), using aqueous sodium deoxycholate and phenol/acetic acid/water to remove intracellular protein, followed by solubilization of starch with aqueous DMSO, was rejected as unsuitable as it also removed soluble β-glucan from the walls. To circumvent these problems, the crude cell wall preparation was washed with 1% SDS in 70% ethanol. As expected, following this treatment, the wall preparation was free of small starch granules (determined microscopically by staining with iodine/potassium iodide, Chayen *et al* 1969), and was also clean with respect to adhering protein (determined microscopically by staining with Fast Green, Fig. 20f; Chayen *et al* 1969). A portion of the wash was freeze-dried, and after hydrolysis in 2.0N H_2SO_4, monosaccharide analysis showed only a small amount of

glucose from the starch granules. This fraction was not examined further.

Because considerable amounts of bran were still present in the endosperm enriched fraction, the differential settling procedure used by Mares and Stone (1973a) to separate residual bran cell walls was insufficient to remove all of the bran contamination from the oat cell wall preparation. A method for removing bran contamination was developed which takes advantage of chemical differences between bran and endosperm cell walls. These differences are highlighted by differential staining with the dye Toluidine Blue O which was used as a microscopic marker for bran contamination. Cell walls derived from bran tissues (aleurone, pericarp, testa) stain bluish-purple with Toluidine Blue O, due to the presence of lignin and/or acidic groups in the cell wall (O'Brien *et al* 1964; Chayen *et al* 1969), while endosperm cell walls, which appear to be chemically neutral, do not bind any of the dye, and remain colourless (Fig 20c).

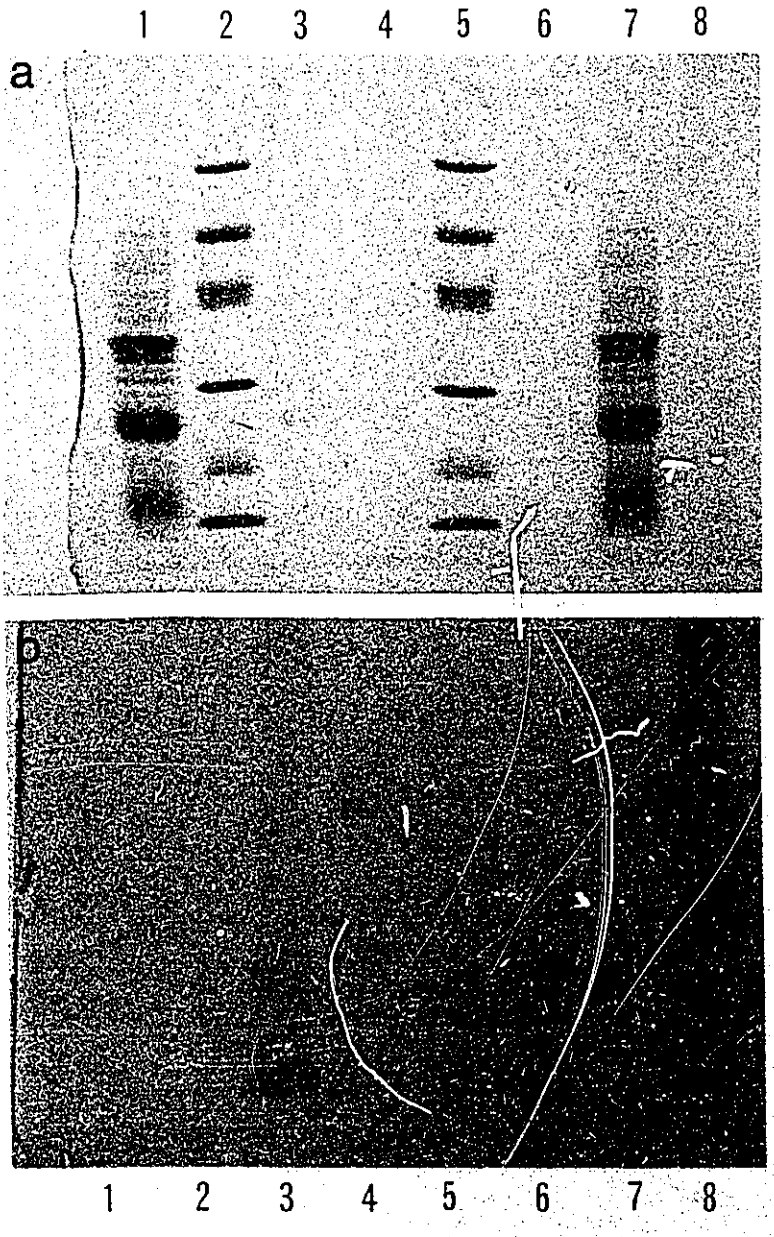
A slurry of anion exchange beads (marketed for column chromatography) was poured into a preparation of crude cell walls in ethanol while stirring, and then the stirring stopped and the contents allowed to settle. The bran cell walls and the anion exchange beads settled fairly rapidly in a pseudo-agglutination reaction (Fig. 20e), leaving the much lighter endosperm cell walls suspended, and easily collected by aspiration. To obtain very pure endosperm cell walls, it was necessary to add a slight excess of anion exchange media, so that the final endosperm cell wall preparation contained approximately 5% contamination of the ion exchange beads. These were identified microscopically by their staining reaction with Acid Fuchsin, and in the case of Sephadex (which was used in the initial isolations), by the presence of (1→6)-linked glucose after methylation analysis of whole endosperm cell walls. Later experiments were performed using a non-carbohydrate based resin, composed of polystyrene. Although the

polystyrene beads appeared intact microscopically after acid hydrolysis, some component was extracted in small amounts which interfered with acetylation and sugar determination using GC. The extracted component did not interfere with similar analyses by HPLC, which was used for the remaining analyses of all acid hydrolysates. Attempts to remove the remaining ion exchange beads by placing the cell wall/bead suspension in an electric field were only partially successful. The cell walls were easily altered by this treatment (as indicated by a change from white to a brownish colour), so the method was not routinely used for preparation of isolated cell walls.

The absence of cytoplasmic protein in the pure endosperm cell wall fraction (indicated by lack of Fast Green staining, Fig. 20f) was confirmed when rigorous extraction procedures failed to extract sufficient protein to be detected by SDS-PAGE of the extract, using the conventional Coomassie blue stain (Fig. 21a), or the more sensitive silver stain (Fig 21b). Samples were first extracted in boiling Phast buffer, and when no protein was detected (Fig. 21a, lanes 3, 6 and 8; Fig. 21b, lanes 1, 2 and 3), a more rigorous overnight pre-extraction in 8M urea followed by the Phast buffer extraction was attempted (Fig. 21b, lanes 4 and 5). Neither of these procedures yielded detectable amounts of protein. A wide range of proteins was obtained from Marion groat flour by extraction in boiling Phast buffer (Fig. 21a, lanes 1 and 7). Cell wall structural proteins are noted for being unextractable from mature primary walls under nondegradative conditions (Mort and Lamport 1977; Lamport 1980; Dey and Brinson 1984; Cassab and Varner 1988), and thus were not expected to be extracted by these procedures.

Figure 21. SDS-PAGE of extracts from oat endosperm cell walls (Marion).
a) Gel stained with Coomassie Blue: lanes 1 and 7, groat flour from Marion oats, 30 min extraction in boiling Phast buffer; lanes 2 and 5, molecular weight standards, 10 min in boiling Phast buffer; lanes 3, 6 and 8, endosperm cell walls, 30 min extraction in boiling Phast buffer; lane 4, blank.

b) Gel stained with silver stain, stained twice for more detail in lanes 6 & 7. Lanes 1-3, endosperm cell walls, 30 min extraction in boiling Phast buffer; lanes 4 and 5, endosperm cell walls, overnight extraction in 8M urea (37°C) followed by 1 hr at 80°C, then 30 mins in boiling Phast buffer; lane 6, overnight digestion of endosperm cell walls in lichenase, then 30 min extraction in boiling Phast buffer; lane 7, lichenase control, 30 min boiling Phast buffer; lane 8, molecular weight standards, 10 min, boiling Phast buffer.


1 2 3 4 5 6 7 8
a
b
1 2 3 4 5 6 7 8

4.3.2 Preliminary characterization of the oat endosperm cell wall

4.3.2.1 ß-Glucan content of endosperm cell walls

In addition to microscopic inspection, the purity of the endosperm cell wall preparations was monitored by assaying ß-glucan content. The β-glucan content of the preparations should increase as cell wall preparations became progressively purer. This was, in fact, found to be the case. The ß-glucan content of whole groats of two cultivars of oats, and of different fractions obtained during the isolation of endosperm cell walls from the groats, is presented in Table 16.

Whole groats of Marion (6.4% β-glucan) contained 40% more β-glucan than whole groats of OA516-2 (3.8% ß-glucan). The tissue removed by abrasion (which consisted predominantly of bran cell walls- aleurone, pericarp and testa- and also most of thetrichomes from the seeds) contained less than 0.5% ß-glucan, probably from the aleurone cell walls (Bacic and Stone 1981a,b; Fulcher 1986). Alternatively, it is possible that the β-glucan measured in these preparations arose from contamination with a small amount of endosperm. As the purity of the cell wall preparation increased, the ß-glucan content increased up to an apparent limit of 75-78%. The sludge from the two preparations consisted of fast-settling material which contained clumps of unbroken cells from both bran and endosperm that retained their cell contents. This fraction was still relatively enriched in β-glucan (~ 26% ß-glucan), because of the large amounts of starch and protein lost during the wet sieving procedure. The ß-glucan content of this fast-settling fraction did not differ significantly between preparations from Marion or OA516-2. The slower-settling fraction contained cell walls from both tissues which were largely devoid of cell contents. The latter, cell wall-containing fraction was resuspended in 2 L of 70% ethanol, and allowed to settle several times. The faster-settling fraction from the

Table 16. ß-Glucan content of fractions from cell wall isolation procedure of 2 cultivars of oats, with high (Marion) and low (OA516-2) contents of ß-glucan in the whole grain. Values are given as % by weight, dry weight basis.

Fraction	Marion	OA516-2
groat flour	6.4	3.8
abraded material	0.1	0.3
sludge from isolation	26.7	26.0
bran-enriched	38.8	38.3
endosperm cell walls	78.3	74.5

second settling procedure was enriched with bran cell walls relative to the final preparation, and had a ß-glucan content of approximately 38% for both Marion and OA516-2. The final endosperm cell wall preparations from OA516-2 and Marion contained roughly twice the amount of ß-glucan (75.4% and 78.3% respectively) that was found in the bran-enriched fraction.

The ß-glucan content of oat endosperm cell walls is somewhat higher than that reported for endosperm cell walls from barley, for which ß-glucan contents of 70-75% were previously reported (Fincher 1975; Forrest and Wainwright 1977; Ballance and Manners 1978). A mixed preparation from oats, containing cell walls from both endosperm and bran was estimated to contain greater than 14% ß-glucan (Aspinall and Carpenter 1984). This preparation would roughly correspond to the bran-enriched fraction in the present study, which was assayed at 38% ß-glucan. The preparation of Aspinall and Carpenter (1984), however, also contained significant amounts of starch which diluted the ß-glucan content of the walls. Starch contamination, and losses of soluble polysaccharides incurred during isolation of cell wall material prevented an accurate estimation of ß-glucan in a similar preparation of mixed cell walls from oats (Selvendran and DuPont 1980).

In contrast to whole groats from Marion and OA516-2, which had large differences in β-glucan content (Marion was 40% higher), the isolated cell walls from both cultivars contained similar amounts of β-glucan, differing by less than 4%. The differences in β-glucan content between the two cultivars are more likely due to differences in cell wall distribution in the endosperm. The micrographs presented in Figure 9c,d (Section 5) show a comparison of the central endosperm from Marion and OA516-2 at the same magnification. The cells in Marion appear to be smaller, so that more cell wall is present

per unit area of the section than in OA516-2. The presence of more cell walls in Marion than in a comparable area of OA516-2 would account for the higher β-glucan content of whole groats from this cultivar.

4.3.2.2 Monosaccharide analysis of oat endosperm cell walls

Determination of the monosaccharide composition is the first step in characterization of cell wall polysaccharides. Although the cell wall represents a mixture of polysaccharides, the monosaccharides present often provide a good indication of the types and proportions of polysaccharides present in the wall. Isolated cell walls were hydrolyzed in both 2.0N and 72% H_2SO_4 to determine monosaccharide composition. The difference in glucose content between the two hydrolysis procedures is considered to be a reasonable estimate of cellulose content in cell wall preparations (Selvendran and DuPont 1980). Although a large proportion of the wall preparations was glucose, the cellulose component was 3% or less. (Actual differences determined from all hydrolyses ranged from 0-3%). Very low values for cellulose content have also been reported in endosperm cell walls of wheat (Mares and Stone 1973a) and barley (Fincher 1975; Forrest and Wainwright 1977; Ballance and Manners 1978). One preparation from oats containing cell walls from both bran and endosperm contained less than 0.5% cellulose (Aspinall and Carpenter 1984), in contrast to a similar preparation which was earlier reported to contain approximately 8% cellulose (Selvendran and DuPont 1980). The discrepancy between the two values may have been due to the loss of water-soluble polymers in the earlier study, which, if not corrected for, could significantly alter the proportions of the components determined in the remaining wall material.

Monosaccharides from hydrolyzed cell walls of Marion were reduced and acetylated, then analyzed by GC. Glucose, xylose, arabinose, mannose and a trace of galactose accounted for approximately 97% of the recovered carbohydrate. The presence of possible breakdown products generated during the production of alditol acetates was inferred from a few small unidentified peaks on the chromatogram, although chromatograms obtained from biological material always show a certain number of unidentified background peaks (Dutton 1973). The most abundant sugar was glucose, which constituted approximately 89% of the isolated walls, after correction for dextran contamination (estimated from methylation data). Arabinose and xylose together made up 6-7% of the wall, and mannose and galactose together accounted for less than 1% of the wall.

To avoid further problems with glucose determinations, polystyrene anion exchange beads were used in all remaining cell wall isolations. An unknown component was extracted from the polystyrene during acid hydrolysis that interfered with detection of alditol acetates by GC. When hydrolysates of cell walls isolated using polystyrene were analyzed by HPLC, a small extra peak was observed in some samples. This was attributed to the unidentified component. The extra peak was well separated from the monosaccharides, however, and did not interfere with detection.

The monosaccharide composition of purified endosperm cell walls, as well as bran-enriched and abraded fractions obtained during isolation procedures of both Marion and OA516-2 cell walls, is shown in Table 17. All three fractions were hydrolyzed in 72% H_2SO_4 for one hour at 4°C, then diluted to 2.0N and hydrolyzedfor a further two hours at 100°C before neutralization and analysis using HPLC. The monosaccharide compositions were similar in each fraction for both cell wall types isolated. Galactose was present in

Table 17. Monosaccharide content of fractions from cell wall isolations from oats using cultivars Marion and OA516-2. Samples were hydrolyzed in 72% H_2SO_4, and the values presented represent the % of the recovered material. For the isolated endosperm cell walls (ECW) 99% of the material was hydrolyzed. In the bran enriched (BE) samples, approximately 9% of the sample was resistant to hydrolysis and not recovered, and approximately 51% of the abraded (ABR) fraction was resistant to hydrolysis.

Sugar	Marion			OA516-2		
	ECW	BE	ABR	ECW	BE	ABR
Glucose	90.0	79.4	15.6	93.5	80.9	14.3
Xylose	4.9	12.1	57.1	3.6	12.0	61.0
Arabinose	3.4	8.3	23.1	2.9	7.1	20.2
Galactose	1.7	tr	4.3	-	-	4.5
Mannose	-	-	-	-	-	-
ara:xyl	0.7	0.7	0.4	0.8	0.6	0.3

such small amounts in endosperm wall preparations from OA516-2, however, that it was not detected at all. The amount of galactose present in unfractionated walls was also too small to be detected by methylation analysis of whole cell walls. Although mannose was detected in alditol acetates analyzed by GC from hyrolyzed Marion cell walls, it was not found in HPLC analyses of underivatized hydrolyzates of whole cell walls from either cultivar.

Approximately 51% by weight of the abraded fraction was resistant to acid hydrolysis, even in 72% H_2SO_4. Of the carbohydrate recovered, approximately 85% was arabinoxylan (calculated from the sum of xylose, arabinose and galactose). The low arabinose:xylose ratio for this fraction indicated a low degree of substitution for the arabinoxylan in this fraction. Glucose made up 14-15% of the abraded fraction, presumably in the form of cellulose, as this fraction contained only 0.5% β-glucan. It is also possible that starch contamination from the endosperm contributed to the glucose content of this fraction as well.

Only 9% by weight of the bran-enriched fraction was resistant to hydrolysis. Of the carbohydrate recovered from this fraction, approximately 80% was glucose. As with the abraded fraction, β-glucan measured in the samples (~ 38%) did not account for all the glucose present. Nevertheless, it is unlikely that cellulose accounts for all of the remaining glucose in this fraction (Selvendran and DuPont 1980; Aspinall and Carpenter 1984). Although no starch determination was performed on this fraction, the contents of the intact cells observed microscopically in this fraction would contribute significant amounts of glucose from starch to the hydrolysate. Nineteen to twenty per cent of the carbohydrate recovered from the bran-enriched fraction was xylose and arabinose (presumably arabinoxylan). The degree of substitution of the arabinoxylan was higher in

this fraction than in the abraded fraction.

The arabinoxylan content of purified endosperm cell walls was much lower (6.5-10%) than for the bran-enriched and abraded fractions. This confirms the low pentosan content in oat endosperm that was previously reported by Henry (1987), who found higher pentosan contents in whole groats than in isolated endosperm tissue from oats. Oats contained lower amounts of pentosan than any of the other cereals included in that study.

Virtually all of the purified endosperm cell walls were hydrolyzed under the conditions used. Glucose accounted for 90-93% of the recovered carbohydrate. The β-glucan content of isolated cell walls, however, was 74-78%. An insoluble, cellulose-like precipitate formed on hydrolysis of β-glucan has been reported in extracts from barley and oats (Henry 1985; McCleary and Glennie-Holmes 1985; Wood *et al* 1991a), and was also observed in the present study (see Section 6.3.2.6). Although McCleary and Glennie-Holmes (1985) claimed that the loss of this material was insignificant, and that addition of cellulase to degrade the insoluble higher d.p. oligosaccharides did not change the β-glucan yield for whole grain samples, it is likely that the effect would be much more noticeable in isolated endosperm cell walls, which have such a large proportion of β-glucan. The β-glucan content of the oat endosperm cell walls was thus probably underestimated in the present study as a result of the loss of these higher d.p. oligosaccharides, and would therefore account for more of the glucose in the cell wall (approximately 80-85%) than was indicated by the enzymatic assay. Small amounts of cellulose and glucomannan would also contribute to the amount of glucose recovered. Although it was not detected microscopically, small amounts of starch might also have been present, and thus contributed to the glucose recovered. This contribution of starch

was noted in determinations of barley endosperm cell wall composition. Ballance and Manners (1978) reported that the cell walls appeared starch-free microscopically, but that when estimated enzymatically, starch accounted for 2.5% by weight of their barley endosperm cell wall preparation.

The ß-glucan and monosaccharide composition of isolated oat endosperm cell walls indicates that β-glucan and arabinoxylan are the main polysaccharide components. Small amounts of cellulose are also present, and probably some glucomannan as well. These results are in close agreement with the reported carbohydrate composition of barley endosperm cell walls, although galactose has not been reported in barley cell walls (Fincher 1975; Palmer 1975; Forrest and Wainwright 1977; Ballance and Manners 1978; Ahluwalia and Ellis 1985).

4.3.2.3 Methylation analysis of endosperm cell walls

A sample of Marion cell walls was methylated by the method of Hakomori (1964). A fraction from the cell wall isolation procedure containing small cell wall fragments (fragments which passed a 48 μm screen but were retained on a 30 μm screen) was used for this experiment, as an earlier report suggested that reduced particle size (10-40 μm) allowed more efficient methylation of whole cell walls (Lomax *et al* 1983). The methylated sample was submitted for gas chromatography-mass spectrometry (GC-MS), and the methylated sugars were identified by GC retention times and MS fragmentation patterns, which were compared with published values (Björndal *et al* 1967; Lindberg 1972; Jansson *et al* 1976; Lomax *et al* 1983; Hellerqvist 1990).

The most abundant methylated sugars were 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylglucose and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucose (corresponding to (1→3)-

and (1→4)-linked glucosyl residues), which together accounted for 85.7% of the material recovered. Dextran contamination in the sample was evident in the presence of a small amount (approximately 2%) of 1,5,6-tri-O-acetyl-2,3,4-tri-O-methylglucose. 1,5-Di-O-acetyl-2,3,4,6-Tetra-O-methylglucose, which represents the non-reducing end of the polysaccharide, accounted for a further 0.9%. The total of these components is lower than the total glucose determined by hydrolysis and monosaccharide analysis of the walls. A small number of mono- and di-methylated sugars, and a small amount of hexose hexaacetate (altogether 6.5% of the total carbohydrate) indicated that the cell walls, even after 2 cycles of methylation, were not completely methylated, probably as a result of incomplete dissolution of the cell wall in the methylation solvent. The mass spectral characteristics and GC retention times of the mono- and di-methylated sugars indicated that they were mainly hexoses, but further identification was not possible. The presence of both (1→3)- and (1→4)-linked glucose in such a high proportion in the cell walls is consistent with the high level of mixed linkage β-glucan determined enzymatically in these samples.

Methylation data also provides further evidence for the presence of arabinoxylan in the cell wall. 1,4-Di-O-acetyl-2,3,5-tri-O-methylarabinose (0.7%) and 1,4,5-tri-O-acetyl-2,3-di-O-methylxylose (2%) corresponding to terminal arabinosyl units and a backbone of (1→4)-linked xylose residues were both detected in the methylated cell walls. Although the presence of 1,3,4,5-tetra-O-acetyl-2-O-methylxylose was expected to complement the terminal arabinose residues, no peak for this derivative was identified. Judging from published relative retention times (Jansson *et al* 1976; Lomax *et al* 1983) it is probable that this peak, if present, was masked by the large 2,3,6-tri-methylglucose peak.

A small amount of 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylmannose (2.2%, (1→4)-linked)

was also detected in the cell walls. Small amounts of glucomannan have been reported in the endosperm cell walls of wheat (Mares and Stone 1973a) and barley (Fincher 1975; Ballance and Manners 1978; Ahluwalia and Ellis 1985). Mannose was also detected in the mixed oat cell wall preparations of Selvendran and DuPont (1980) and Aspinall and Carpenter (1984).

Because of undermethylation and incomplete identification of the undermethylated hexoses, it was not practical to attempt a stoichiometric comparison of the data. Nevertheless, the combined results from ß-glucan, monosaccharide and methylation analyses indicate the presence of mixed linkage ß-glucan, arabinoxylan and small amounts of glucomannan and cellulose in the oat endosperm cell wall.

4.3.2.4 Amino acid analysis of endosperm cell walls

As mentioned previously, no detectable protein was extractable from the purified endosperm cell walls. Digestion of the cell walls with lichenase (which would remove a large portion of the wall and possibly make the proteins more accessible) was also unsuccessful in solubilizing significant amounts of wall protein (Fig 21b, lane 6). Cell wall structural proteins were previously reported to remain insoluble even after removal of the polysaccharide components of the wall with anhydrous hydrogen fluoride (Mort and Lamport 1977). After acid hydrolysis of the walls, however, amino acids were detected in the hydrolyzate indicating a total protein content in the walls of 1.7%. The amino acid composition of Marion endosperm cell walls is presented in Table 18, and, for the purpose of comparison, the amino acid composition of whole groat flour from Marion. The total protein of groat flour calculated from amino acid recovery accounted for 19.1% of the dry weight. This value was consistent with Kjeldahl determinations of protein

content in groat flour from Marion oats (data not shown). Hydroxyproline was not detected in the cell walls or in the groat flour. In comparison with the groat flour, the isolated cell walls contained higher amounts of alanine, glycine, lysine and tyrosine. The groat flour contained a higher proportion of glutamine and glutamic acid (reported as glutamic acid), asparagine and aspartic acid (reported as aspartic acid), and valine than the isolated cell walls. The higher proportion of glutamic acid in the groat flour is a reflection of the glutelin content in oats (Peterson and Brinegar 1986).

Mares and Stone (1973a) reported a protein content of 14-15% in wheat endosperm cell walls. They also pointed out that at least half of the protein in their cell wall preparation appeared to be of cytoplasmic origin, and was identified with irregularly shaped particles observed on the surface of cell walls in electron micrographs. The reported protein content of barley endosperm cell walls was also higher than the value obtained for oat endosperm cell walls in the present work. Ballance and Manners (1978) reported approximately 5% protein in barley endosperm cell walls, although some of this was believed to be cytoplasmic protein, judged from microscopic evidence. Less than 1% nitrogen was reported in barley endosperm cell walls by Fincher (1975), and applying the conversion factor of 6.25, the cell walls can be estimated to contain approximately 6% protein. Selvendran and DuPont (1980) reported 6.1% protein in their mixed oat cell wall preparation.

Although the amount of protein determined in the mixed oat cell wall preparation (Selvendran and DuPont 1980) was higher than the protein content of the oat endosperm cell walls isolated in the present study, the amino acid composition (%) of the two preparations was very similar. The major difference between the two preparations was

Table 18. Amino acid profile of Marion endosperm cell walls (ECW), with the amino acid profile of groat flour (GF) from Marion for comparison. Amino acid values are given as mole% (Average of 2 determinations).

Amino acid	ECW	GF
Ala	10.1	8.1
Arg	2.5	3.0
Asp+Asn	8.3	9.7
Cys	-	1.05
Glu+Gln	14.9	22.65
Gly	10.7	9.2
His	-	-
Hyp	-	-
Ile	4.9	4.5
Leu	8.6	8.3
Lys	5.9	3.9
Met	1.1	1.6
Phe	4.2	3.8
Pro	6.0	5.8
Ser	7.5	7.3
Thr	4.9	4.7
Trp	nd	nd
Tyr	3.8	2.7
Val	7.1	8.4

nd: not determined

the presence of a small amount of hydroxyproline and histidine (0.7% and 1.3% respectively) in the mixed cell walls, where none was detected in endosperm cell walls. Hydroxyproline was not detected in wheat or barley endosperm cell walls (Mares and Stone 1973b; Fincher 1975). The higher protein content of the mixed oat cell wall preparation could be partly due to the loss of water soluble polymers during cell wall isolation, which would increase the proportions of the remaining components in the wall. The present work confirms the observation of Selvendran and DuPont (1980) that the amino acid composition of the cell walls is in general similar to that of soluble cytoplasmic proteins, although some differences, as observed above, are apparent.

4.3.2.5 Phenolic acids in oat endosperm cell walls

Phenolic acids have been reported in the aleurone and germ cell walls of wheat, barley and oats (Fulcher *et al* 1972; Smart and O'Brien 1979; Fulcher and Wong 1980; Fulcher 1986). Phenolic components have also been detected in the endosperm cell walls of wheat and barley (Mares and Stone 1973b; Fincher 1975, 1976; Ahluwalia and Fry 1986), and in mixed cell wall preparations from oats (Selvendran and DuPont 1980; Hartley 1987). The most abundant phenolic acids found in both vegetative and grain tissues in the Gramineae are ferulic and *p*-coumaric acids (Durkee and Thivierge 1977; Hartley and Keene 1984; Nordkvist *et al* 1984; Shibuya 1984; Eraso and Hartley 1990; Hartley *et al* 1990).

In the present work, phenolic acids were extracted and determined in isolated endosperm cell walls, as well as in the bran-enriched fraction and in the material produced in the initial abrasion procedure. Ferulic and *p*-coumaric acids were the dominant phenolics extracted from all three fractions. Although both *Z*- (cis-) and *E*-

(trans-) ferulic and *p*-coumaric acid were present in the final extracts, these were converted and reported as *E*-ferulic acid and *E*-*p*-coumaric acid, as these are the dominant forms in plants (Harris and Hartley 1980). Once extracted, both phenolic acids are very easily isomerized by exposure to ultraviolet light (Kahnt 1967; Hartley and Jones 1975). Small peaks indicated the presence of other compounds, but these were quantitatively much less and were not identifiable with any of the standards tested. Total phenolics as reported in this thesis are the total of ferulic and *p*-coumaric acids determined.

The phenolic acid components of different fractions from isolation of endosperm cell walls of Marion and OA516-2 are shown in Table 19. When these values were converted to a percentage (dry weight) basis, the total phenolic acid content of purified endosperm cell walls was 0.005-0.015%. As the amount of bran tissue in the wall preparations increased, the content of phenolics increased also: 0.12-0.18% in the bran enriched fraction, and 0.54-0.65% in the abraded fraction. Ferulic acid was the predominant phenolic component in all fractions, particularly in the bran-enriched and abraded fractions. In contrast to comparisons of β-glucan content or monosaccharide composition, which were similar for cell walls from both Marion and OA516-2, differences were observed in the levels of phenolic acids between the two cultivars. In all fractions, the total phenolic acid content was higher in OA516-2 than in Marion, although the magnitude of the difference decreased as bran content increased. If this difference were confined to the endosperm cell walls, it could be suspected that OA516-2 was contaminated by non-endosperm cell walls. This, however, was not the case: both the ferulic and *p*-coumaric acid contents are higher for OA516-2 than for Marion for all three fractions assayed, with the largest difference being observed in the endosperm cell walls.

Table 19. Phenolic acids in fractions from isolated cell walls of Marion and OA516-2. Values shown represent ferulic acid as the total of *E*- and *Z*-ferulic acids, and *p*-coumaric as the total of both isomers as well, in µg/g cell wall.

Fraction	ferulic	*p*-coumaric
MARION		
endosperm	31	20
bran-enriched	1108	124
abraded	4006	1413
OA516-2		
endosperm	121	31
bran-enriched	1565	189
abraded	4742	1767

These differences in phenolic acid content of the cell walls may reflect other plant characteristics (e.g. germination: OA516-2 carries a dormancy trait and Marion does not; or disease or pest resistance), but there is not enough information available at this time to draw any conclusions regarding the role of phenolics in the cell wall.

The total phenolic acid content of oat endosperm cell walls was lower than the amount reported for barley endosperm cell walls (0.05%, Fincher 1976; 0.06%, Ahluwalia and Fry 1986). The total phenolic content of mixed oat cell walls was much higher (0.81%, Selvendran and DuPont 1980; 0.75%, Hartley 1987). The high phenolic content of the mixed cell wall preparations was undoubtedly due to the presence of non-endosperm cell walls, which, as the present study shows (Table 19), contain much higher levels of phenolics than the endosperm cell walls. Higher concentrations of phenolics were also observed in the bran than in the endosperm of barley (Nordkvist *et al* 1984). Confirmation of the presence of phenolics in the endosperm cell walls (i.e. not arising from bran contamination) was obtained by observation of autofluorescence in the isolated endosperm cell walls (Fig. 22). Similar autofluorescence has been reported in isolated endosperm cell walls of barley (Fincher 1976). When viewed in thin sections, the autofluorescence in the oat endosperm cell walls appears to be localized around the outside of the cell wall and possibly in the middle lamella (Fig 23a,b). This is more clearly seen in regions of the kernel where the cell wall is thicker (eg. adjacent to the depleted layer, Fig. 23a) than in the central endosperm, where the cell walls are much thinner (Fig. 23b).

4.3.2.6 Fractionation of endosperm cell walls

The endosperm cell walls isolated from high and low ß-glucan oats (Marion and OA516-2) were subjected to sequential fractionation with hot water, lichenase digestion, and xylanase digestion. This protocol was designed to minimize or eliminate random and uncontrolled cleavage of various types of bonds that can occur during a traditional chemical fractionation of cell walls (Kato and Nevins 1984) so that the fractions obtained would be more representative of the components of the native cell wall.

Hot water extraction: At room temperature (21°C), 46.4% of Marion endosperm cell walls were soluble in water. Extraction at 65°C solubilized a further 28.6% of the cell wall, for a total of 75% of the endosperm cell wall that was soluble in water. In single-step extractions of endosperm cell walls in water at 65°C, 75-80% of the wall was found to be soluble, which was consistent with the results obtained for the two-step extraction at 21° and 65°C. All further aqueous extractions of cell walls from both cultivars were performed at 65°C.

The monosaccharide composition of the 65° aqueous extract indicated that it was composed primarily of ß-glucan (98.4% glucose) with a small amount of arabinoxylan (1.6%). A trace of mannose was also detected in this fraction. Only a third (32-34%, Ballance and Manners 1978) to one half (46-49%, Forrest and Wainwright 1977) of the barley endosperm wall was extractable at 65°C. The 65° extract from barley endosperm cell walls contained a higher proportion of arabinoxylan (10-12%) and a lower proportion of ß-glucan (88-90%; Forrest and Wainwright 1977; Ballance and Manners 1978) than was found in the hot water extract from oat endosperm cell walls. Mannose was also present

Figure 22. Fluorescence micrograph showing autofluorescence in isolated oat endosperm cell walls. Cell walls were viewed dry on slides, with excitation at 365 nm, emission >420 nm.
Magnification X16.

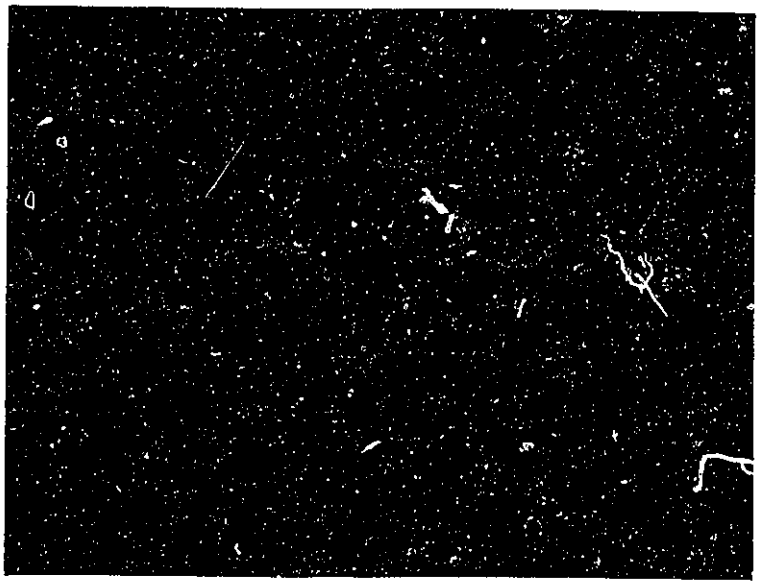
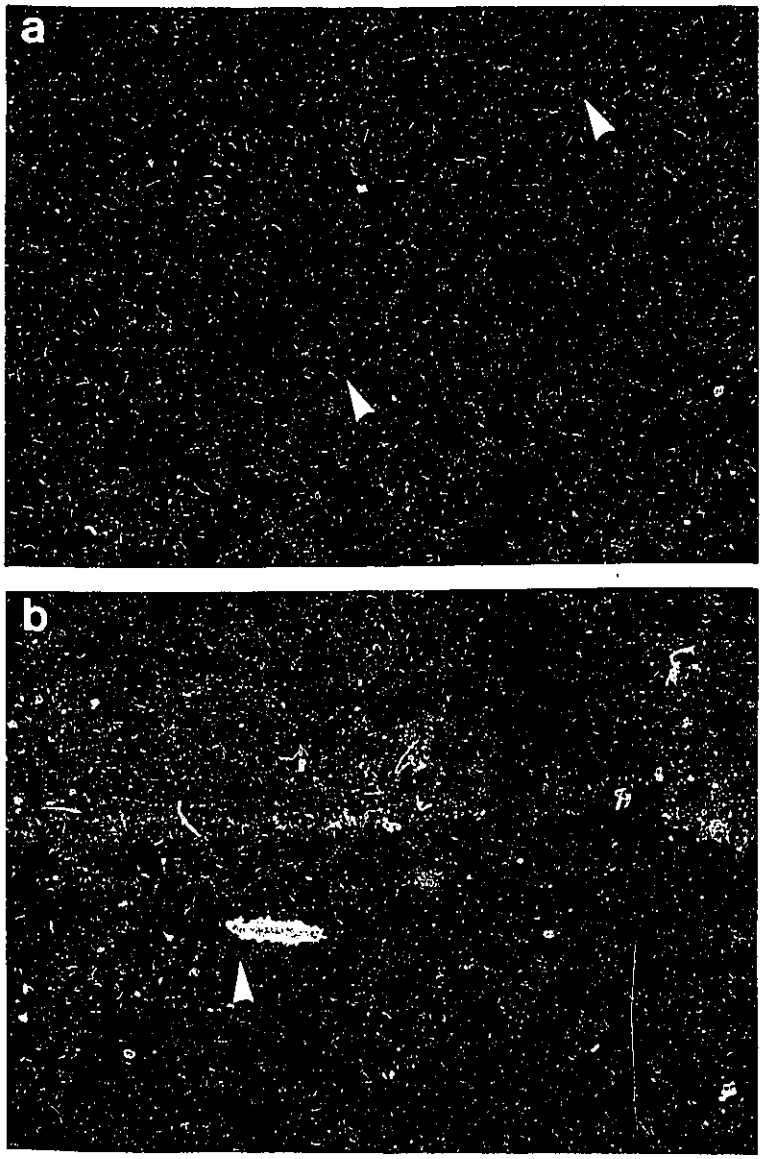


Figure 23. Fluorescence micrographs showing autofluorescence in thin sections (2 μm) of GMA-embedded oats. Excitation at 365 nm, emission >420 nm. a) Autofluorescence in the area next to the germ. Note blue autofluorescence between cells (arrows) which represents phenolics in the outer layer of the cell wall and/or the middle lamella, between the cell walls. b) Autofluorescence in the thin cell walls of the starchy endosperm (arrow). Magnification in a and b X100.


a
b

in small amounts in aqueous extracts from barley at 40° (Fincher 1975; Ballance and Manners 1978) and 65° (Ballance and Manners 1978).

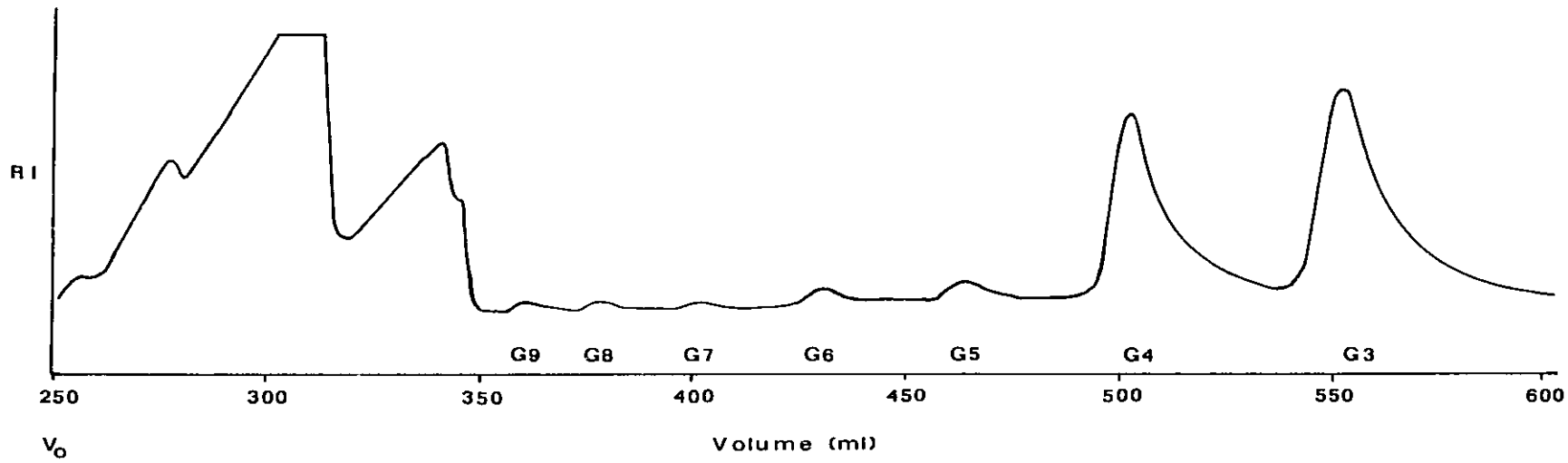
Hot water extracts of the endosperm cell walls were digested with lichenase, and the oligosaccharides produced fractionated on BioGel P-2. During lichenase digestion, a small amount (2-7%) of the original extract came out of solution as a fibrous, white, cellulose-like precipitate. A higher proportion of this material was recovered from hot water extracts of Marion cell walls than from OA516-2 cell walls. The precipitate was hydrolyzed in 2.0N H_2SO_4, and HPLC analysis of the hydrolyzate showed primarily glucose, with traces of arabinose and xylose also present. The remaining arabinoxylan in the hot water extract eluted in the void volume of the BioGel P-2 columns after lichenase digestion.

A similar cellulose-like precipitate was previously noted in lichenase digests of ß-glucan extracted from barley (Woodward *et al* 1983; McCleary and Glennie-Holmes 1985) and from oats as well (Wood *et al* 1991a). This precipitate was apparently of a small enough proportion that it did not affect the total ß-glucan determination in whole grain samples (McCleary and Glennie-Holmes 1985). In isolated oat endosperm cell walls which have been assayed to contain 74-78% ß-glucan, however, the insoluble precipitate would represent a much more significant proportion of the total. If it is assumed that a similar proportion of unextracted ß-glucan in the wall is unavailable for assay, the actual ß-glucan content of the wall approaches 85%. The BioGel P-2 fractionation patterns of both soluble and insoluble ß-glucan in the endosperm cell wall are very similar. This similarity in oligosaccharide fractionation patterns suggests that there is little difference structurally between the soluble and insoluble ß-glucan fractions, giving credence to the assumption upon which the above calculation of total ß-glucan in the wall is based.

In all fractionations of enzyme digests (lichenase and xylanase) on BioGel P-2, large asymmetrical peaks were observed at the void volume of the columns (Figs. 24, 27 and 28). These peaks were also observed when control samples containing only buffer were applied to the columns. Inorganic salts were previously reported to elute with skewed profiles at the void volumes of gel filtration columns of various compositions (Pecsok and Saunders 1968; Neddermeyer and Rogers 1968). Small amounts of higher molecular weight polymers co-eluted with the buffer salts, as demonstrated by the presence of small amounts of glucose, xylose and arabinose in these fractions. While it was possible to reduce the magnitude of these peaks by desalting samples on a small column prior to application on the BioGel P-2, the desalting process resulted in loss of approximately 14% of the sample. This loss was considered unacceptable, particularly for the xylanase digests, in which the amount of material recovered was already quite small. In addition, the major oligosaccharides eluted well past the buffer peaks, and were clearly discernible.

The BioGel P-2 fractionation pattern of lichenase digests of hot water extracts was similar for both Marion and OA516-2 cell walls. A typical fractionation is presented in Fig. 24, which shows the series of gluco-oligosaccharides generated by lichenase digestion of the hot water extract of OA516-2 cell walls. The fractions represented in each peak were pooled, lyophilized, and the glucose content of each fraction assayed after ß-glucosidase digestion by the glucose oxidase/peroxidase reaction. Tri- and tetrasaccharides accounted for approximately 90% of the gluco-oligosaccharides present. The calculated tri-:tetra- saccharide ratios for the hot water fraction from several digests of extracts from both Marion and OA516-2 cell walls ranged from 1.45 to 1.85, which is lower than the ratios previously determined for total ß-glucan in unextracted samples. In

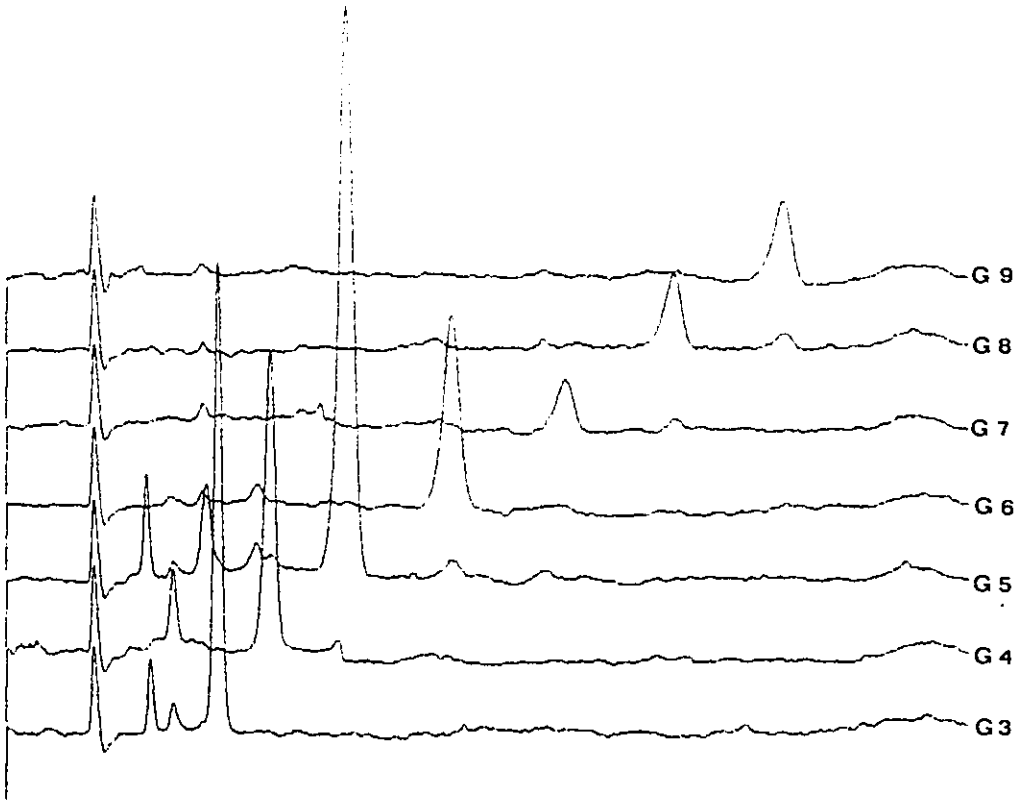
Figure 24. Chromatogram showing separation of oligosaccharides from lichenase digestion of hot water extract of endosperm cell walls from OA516-2. RI, refractive index; V_o, void volume; G3 etc., gluco-oligosaccharide of degree of polymerisation 3, etc.


RI
G9
G8
G7
G6
G5
G4
G3
250
300
350
400
450
500
550
600
V_o
Volume (ml)

addition to tri- and tetrasaccharides, small amounts of disaccharide were sometimes present, and regularly separated peaks indicated the presence of gluco-oligosaccharides of a degree of polymerization (d.p.) from 5 to 8, and sometimes 9 glucose units. The presence of glucose in the fractions containing the buffer peaks suggests that some gluco-oligosaccharides of d.p. 10 may also be present. It is likely that the ß-glucan in the original hot water extract contained even longer runs of gluco-oligosaccharides which hydrogen-bonded together and became insoluble when the ß-glucan was cleaved, producing the cellulose-like precipitate mentioned above. Examination of the insolubles generated by lichenase digestion of ß-glucan extracted from barley indicated that oligosaccharides up to a d.p. of 20 were present (Woodward *et al* 1983b).

A series of gluco-oligosaccharides collected from the lichenase digestion of a hot water extraction of cell walls of OA516-2 were rechromatographed individually by HPLC using PAD. Although the peaks on the BioGel P-2 chromatogram appeared, for the most part, well separated (Fig. 24), HPLC analysis showed that in many cases, the primary gluco-oligosaccharide was contaminated by small amounts of one or more oligosaccharides of higher or lower d.p.(Fig. 25). The peaks in Figure 25 are not representative of the actual proportions of oligosaccharides present in the original separation, but rather represent rough dilutions intended to register within the sensitivity limits of the detector used (PAD). It is likely that small amounts of the oligosaccharides hydrogen-bond to each other, and are thus eluted from the gel permeation columns together. The conditions for ion exchange in the HPLC column would cause dissociation of these bonds, and allow the oligosaccharides in each fraction to separate completely. Small amounts of tri- and tetrasaccharide were observed in the chromatograms from several of the higher d.p. oligomers (Fig. 25), and this probably accounts for the

Figure 25. Overlay of 7 individual chromatograms from HPLC analysis of oligosaccharides G3-G9 isolated from lichenase digest of hot water extract of oat endosperm cell walls shown in Fig. 24. Peak areas are not representative of the actual amounts present in the original digest, but were roughly diluted to fall within the sensitivity of the PAD detector, and are intended for qualitative analysis only.


G 9
G 8
G 7
G 6
G 5
G 4
G 3

differences in tri-:tetrasaccharide ratios observed between whole grain extracts and isolated cell walls.

Lichenase digestion of hot water residue: The residue from hot water extraction was approximately 20% of the starting wall material, for both Marion and OA516-2 cell walls. This residue was enriched in arabinoxylan (~26%) relative to the whole cell wall (~7%). The arabinose:xylose ratio in this fraction was 0.72. The most abundant polysaccharide, however, was still ß-glucan: 10-14% of the original wall, which translates to between 50% and 70% of the hot water residue. Cellulose and glucomannan accounted for the remaining glucose in this fraction (a total of 74% glucose in the hot water residue). Although the hot water residue represented only about 20% of the original wall, the walls retained much of their physical appearance. Figure 26 shows the cell walls stained with Calcofluor during different stages of extraction. Calcofluor binding was reduced in the hot water residue (Fig. 26b) relative to the unextracted cell walls (Fig. 26a). The ion exchange beads, which were slightly autofluorescent, also bound the dye, and the fragmented beads can be seen as the very bright fluorescent particles in the lichenase (Fig. 26c) and xylanase (Fig. 26d) residues.

Digestion of the hot water residue with lichenase solubilized a further 10-14% of the original wall material. The BioGel P-2 fractionation pattern of lichenase digests of the hot water residue was similar for both Marion and OA516-2 cell walls. These fractionation patterns were also very similar to the patterns obtained after lichenase digestion of the hot water extracts. A typical fractionation, in this case for lichenase digestion of the hot water residue of Marion cell walls, is presented in Figure 27. The fractions for each peak were pooled and lyophilized, and the glucose content of each fraction after ß-glucosidase

Figure 26. Micrographs showing oat endosperm cell walls at different stages of fractionation. Preparations in plates a-d were stained with Calcofluor, and viewed at 365 nm (emission >420 nm), magnification X16. a) Whole, unfractionated endosperm cell walls. b) Endosperm cell walls after hot water extraction. c) Endosperm cell walls (large arrows) and some residual anion exchange resin (small arrows) after hot water extraction and lichenase digestion of hot water residue. d) Endosperm cell wall (large arrow) and particles of anion exchange resin (small arrow) after sequential hot water extraction, lichenase digestion, and xylanase digestion. e) Same preparation as (d) stained with Congo Red and viewed with bright field optics (magnification X100).

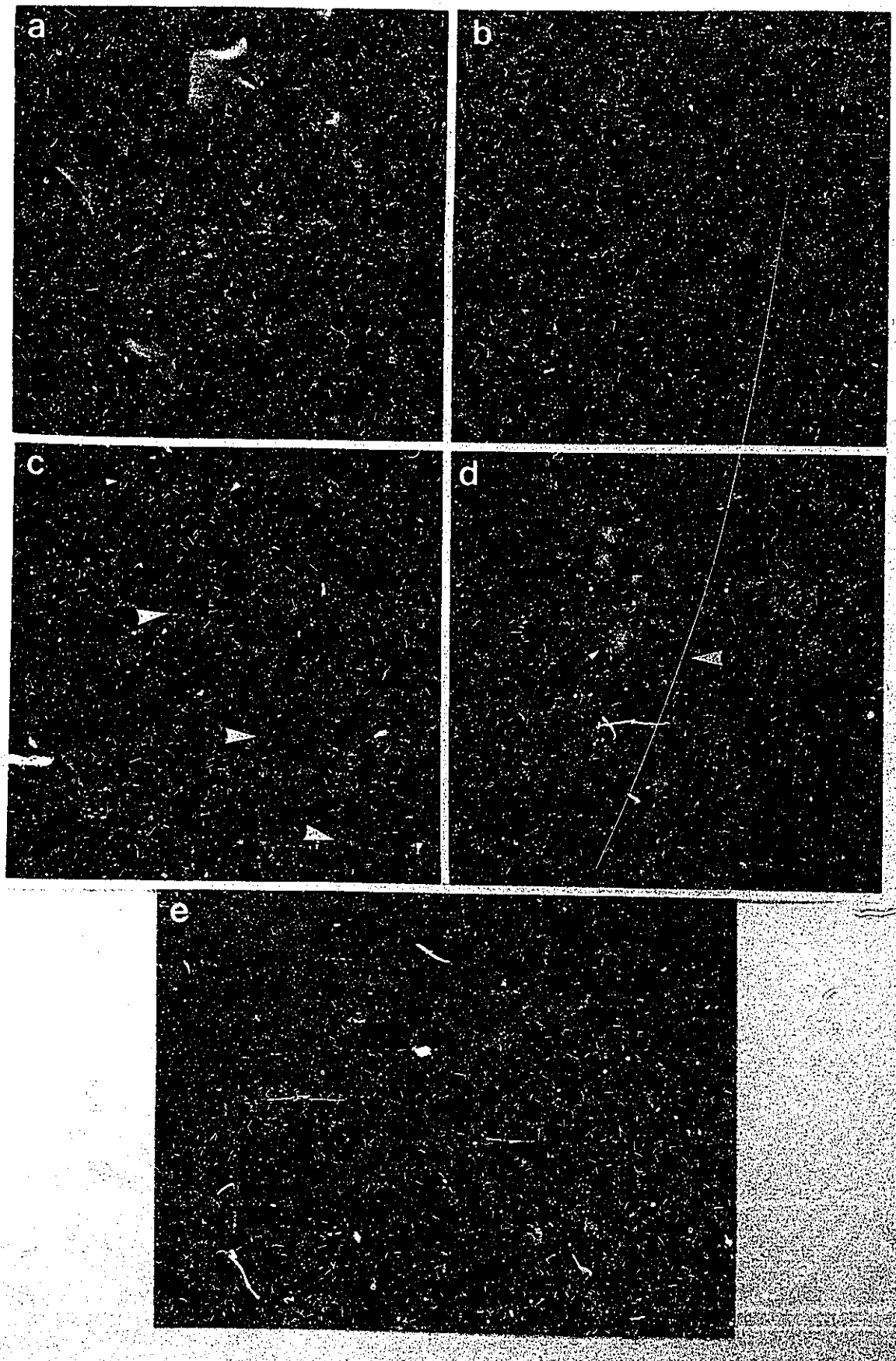
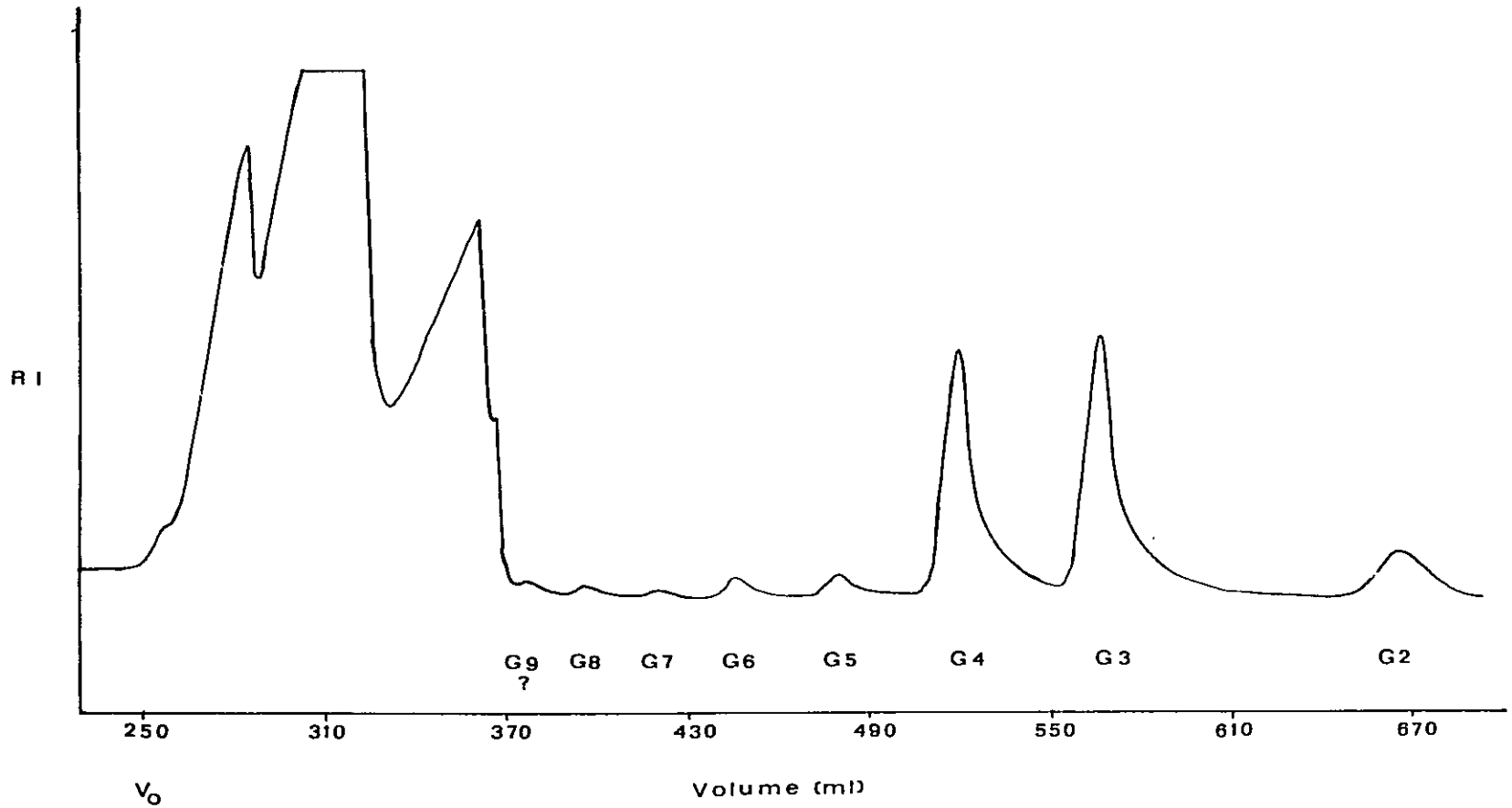

a
b
c
d
e

Figure 27. Chromatogram showing separation of oligosaccharides from lichenase digestion of hot water residue of endosperm cell walls from Marion. RI, refractive index; V_o, void volume; G3 etc., gluco-oligosaccharide of degree of polymerisation 3, etc.


RI
G9
?
G8
G7
G6
G5
G4
G3
G2
250
310
370
430
490
550
610
670
V_o
Volume (ml)

digestion determined by the glucose oxidase/peroxidase reaction. As in lichenase-digested hot water extracts from the cell walls, tri- and tetrasaccharides accounted for approximately 90% of the material recovered from the columns. A similar proportion of the total glucan was previously reported to be represented by tri- and tetrasaccharides in barley (Woodward *et al* 1983b) and in oats (Wood *et al* 1991a). Small amounts of disaccharide were also recovered in some cases. Peaks representing gluco-oligosaccharides of d.p. 5-8 were clearly discernible in all digests of hot water residues. Wood *et al* (1991a) reported that gluco-oligosaccharides from di- up to only hexasaccharide were present in lichenase digests from whole oats and oat brans. Results reported in Section 3, however, indicate that higher d.p. oligosaccharides would have been present in such low quantities in digests from whole oats that they would not have been detected. Small amounts of glucose were also detected at the void volume, and were presumed to represent gluco-oligosaccharides of d.p. 9 and possibly d.p. 10, although the shape and definition of the peaks was masked by the large buffer salt peaks. The fractions under the highest molecular weight peak contained traces of arabinose and xylose, indicating that additional arabinoxylan was solubilized during lichenase digestion.

The tri-:tetrasaccharide ratios (1.63-1.86) were lower than the ratios obtained for total ß-glucan in whole flour digests. Preliminary studies using HPLC to examine oligosaccharides from the lichenase digested hot water extract isolated by gel permeation suggest that the altered ratios are due to the elution of a proportion of the tri- and tetrasaccharides with higher d.p. oligosaccharides (see Fig. 25). Although the fractionation protocol used did not permit detection of insoluble gluco-oligosaccharides that may have been generated during lichenase digestion of the hot water residue, the

similarity of the soluble digestion products from the hot water extract and the hot water residue suggests that such fragments were probably present, and remained with the insoluble residue after lichenase digestion.

Xylanase digestion of lichenase residue: After lichenase digestion of the hot water residue, only 6-10% of the original wall material remained. Hydrolysis of the residue in 72% H_2SO_4 gave an average composition of 34.1% glucose, 30.0% xylose, 18.4% arabinose, 1.6% galactose, and 16.3% mannose. The monosaccharide composition of this residue was similar for both cell wall types, although for Marion the amount of glucose was slightly higher, and the amount of mannose somewhat lower than for OA516-2. This result may be a reflection of the higher proportion of insolubles that was observed after lichenase digestion of hot water extracts from Marion cell walls as compared to extracts from OA516-2. Given the apparent structural similarity of the soluble and insoluble fractions, it is possible that the insoluble fraction from Marion would also yield a slightly higher proportion of insoluble oligosaccharides than the same fraction from OA516-2, thus contributing to the observed difference in glucose content of the lichenase residues. The remainder of the glucose in this fraction (total of 34% in the residue after lichenase digestion) was present as approximately equal amounts of cellulose and glucomannan (estimated from the mannose content of 16% for this fraction). The lichenase residue was enriched in arabinoxylan (~50%) relative to the hot water residue (26%) by a factor of almost 2.

Microscopically, the cell walls appeared to retain some structure, as evidenced by the "ghosts" seen in Fig. 26c. This is in contrast to the observations of Forrest and Wainwright (1977), who reported that no cell wall structure was discernible

microscopically after removal of water-insoluble ß-glucan. The binding of Calcofluor to the lichenase digested walls was much reduced in comparison with unfractionated walls, or hot water extracted walls (Fig. 26), indicating the loss of ß-glucan from these walls.

The residue after lichenase digestion of the hot water residue was digested with xylanase, solubilizing a further 2-5% of the original cell wall preparation. Although the lichenase residue was enriched in arabinoxylan relative to the native walls, the amount present was nevertheless very small, and the sensitivity of the refractive index detector had to be increased by several orders of magnitude in order for the oligosaccharides which were separated by BioGel P-2 chromatography to be observed. As pentose-oligosaccharide standards were unavailable at the time these experiments were performed, the gluco-oligosaccharides which were produced upon lichenase digestion were used as rough standards to estimate the size of the oligosaccharides produced on xylanase digestion. Although this entailed comparing elution volumes of oligosaccharides composed of hexoses with elution volumes of oligosaccharides composed primarily of pentoses, this was considered a reasonable approach, as glucose and xylose co-eluted as a split peak from the BioGel P-2 (not shown). The fractions for each peak were pooled, freeze-dried, and hydrolyzed in 2.0 N H_2SO_4 for analysis using HPLC, as the quantities of oligosaccharides thus isolated were insufficient for methylation analysis.

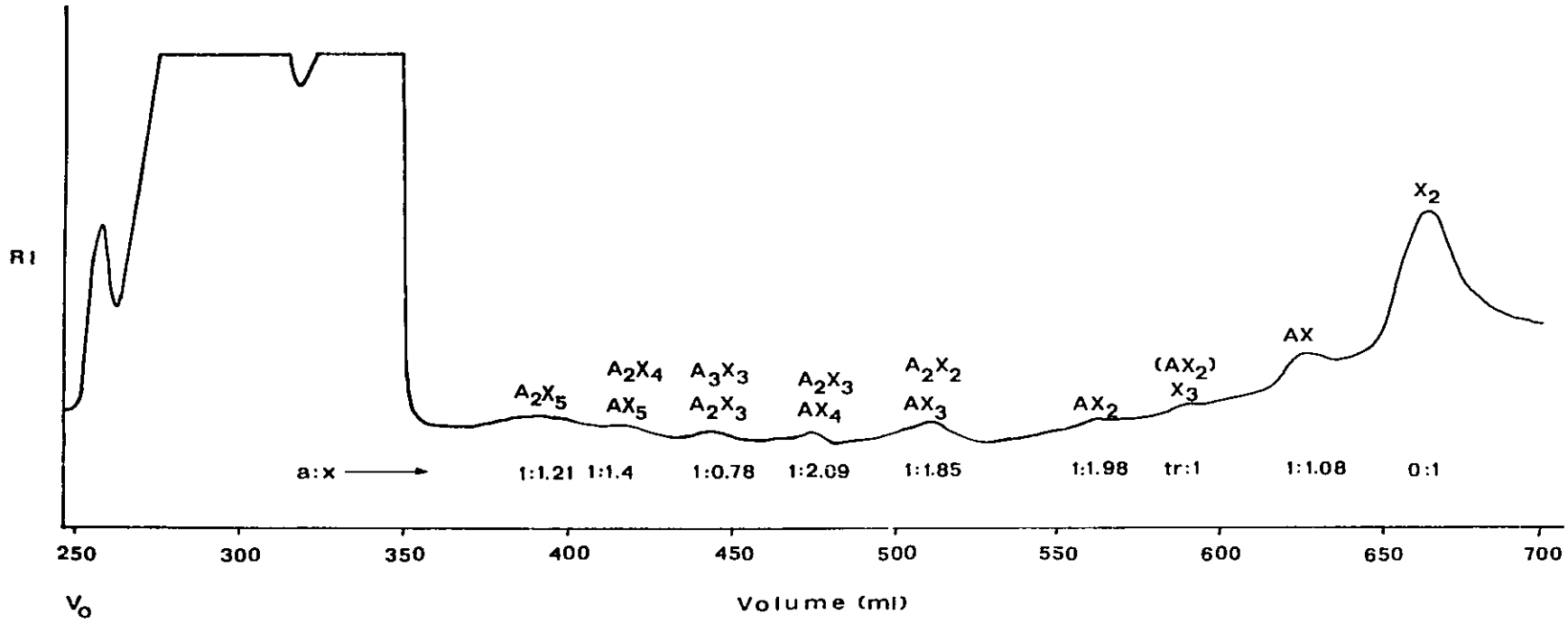
Chromatograms obtained on fractionation of xylanase digests of the lichenase residue (Fig 28) showed a pronounced baseline drift when compared with the fractionation of lichenase digests (Figs. 24 and 27), all of which had very flat, stable baselines. A trial digestion of xylan from oat spelts using the xylanase preparation also had a flat, stable baseline during fractionation on BioGel P-2 (not shown). The elution volume of the peaks from the xylanase digest was, in some cases, within the range of

volumes measured for 2 different gluco-oligosaccharides. Almost all of the fractions collected contained significant amounts of glucose in addition to the xylose and arabinose expected from digestion of arabinoxylan.

A chromatogram showing the fractionation of a xylanase digest of the lichenase residue from endosperm cell walls of OA516-2 is shown in Figure 28. Outside of the buffer peaks, which also contain some polymeric arabinoxylan, the largest peaks can be seen in the regions corresponding to mono- and disaccharides. Smaller peaks can be distinguished in regions corresponding to tri-, tetra-, penta-, hexa- and heptasaccharides. While there was a possibility of some carryover of oligosaccharides between peaks as was observed for the lichenase digest, the changing ratio of arabinose:xylose suggested that the peaks contained different arabinoxylan fragments. Indicated on the figure are the oligosaccharides postulated to be present in each peak based on monosaccharide composition and ratios, and elution volumes. Comparison with published chromatograms for xylanase digestion of arabinoxylan from oat spelts (John and Schmidt 1988) was also helpful in peak assignment.

The assignments of the peaks on the chromatogram from the xylanase digest are very tentative, and complicated by the presence of glucose in almost all of the fractions. The presence of glucose in these fractions could be interpreted to indicate that arabinoxylan is covalently bonded to a glucose-containing polysaccharide (which would have to be ß-glucan, since any arabinoxylan attached to cellulose would remain with the insoluble polymer). Alternatively, and in view of the separation behavior of the gluco-oligosaccharides from the lichenase digests, more probably, a small proportion of the gluco-oligosaccharides produced during lichenase digestion hydrogen-bonded to areas of arabinoxylan, and were released and co-eluted with pentose-oligosaccharides after

Figure 28. Chromatogram showing separation of oligosaccharides from xylanase digestion of lichenase residue of endosperm cell walls from OA516-2. RI, refractive index; V_o, void volume; a:x, arabinose:xylose ratio; A, X, Gal and G signify the presence of arabinose, xylose, galactose (trace only) and glucose in the peaks indicated, subscripts indicate approximated proportions of sugars calculated from monosaccharide contents and a:x ratios.


RI
A_2X_5
A_2X_4
AX_5
A_3X_3
A_2X_3
A_2X_3
AX_4
A_2X_2
AX_3
AX_2
(AX_2)
X_3
AX
X_2
a:x
1:1.21
1:1.4
1:0.78
1:2.09
1:1.85
1:1.98
tr:1
1:1.08
0:1
250
300
350
400
450
500
550
600
650
700
V_o
Volume (ml)

xylanase digestion. This view is supported by the presence of peaks with the same retention times as cellotriose and cellotetraose in an aliquot of a xylanase digest that was analyzed using HPLC (not shown). As the separation system of the HPLC used ion exchange rather than gel permeation, co-elution of gluco-oligosaccharides with pentose-oligosaccharides was unlikely.

The final residue, after xylanase digestion of the lichenase residue ranged between 1 and 9% of the original wall, averaging about 3.5%. In general, Marion cell walls had a higher proportion of final residue than walls from OA516-2. As mentioned previously, this might be a reflection of the higher proportion of insolubles generated during lichenase digestion of the hot water residue. The carbohydrate composition of this residue was similar for both cell wall types. For Marion cell walls, the residue contained 52.9% glucose, 26% mannose, 16.3% xylose, 4.8% arabinose, and a trace of galactose. The final residue from OA516-2 cell walls contained 49.4% glucose, 34% mannose, 12.3% xylose, 4.3% arabinose and a trace of galactose. The monosaccharide composition of the final residue indicates the presence of mostly cellulose and glucomannan totalling approximately 69%, although this figure has not been corrected for the presumed presence of insoluble gluco-oligosaccharides generated during lichenase digestion. A small amount of arabinoxylan (16.6%) is also present. The low degree of substitution (arabinose:xylose <0.5) of the arabinoxylan in this fraction would permit a greater degree of hydrogen bonding to the cellulose and glucomannan than would be possible for the more soluble arabinoxylans, which would be prevented from close association with the microfibrils by the presence of arabinose side-chains.

The final residue was also enriched in ion exchange media, since the beads, although physically broken up during the fractionation process, were otherwise

unaffected. This is seen as a mass of brightly fluorescent particles in Fig. 25d. Also visible is the shape of a cell wall, which has bound a small amount of Calcofluor, and fluoresces faintly. The physical structure of the digested remnants of the cell walls is more clearly visible at a higher magnification with the assistance of Congo Red (Fig. 26e).

Lichenase digests of both soluble and insoluble ß-glucans from the endosperm cell walls of either Marion or OA516-2 produced similar oligosaccharide patterns when fractionated using BioGel P-2. The quantity of ß-glucan present in the two fractions was very different, however, with the majority of the glucan in the wall being soluble. The similarity of the oligosaccharide composition of the soluble and insoluble ß-glucans in the endosperm cell wall suggests that the insoluble fraction is probably held in the wall matrix by entanglement and hydrogen-bonding with the microfibrils that make up the wall skeleton and with other matrix components (primarily arabinoxylan) rather than by covalent bonding. Entanglement with insolubie microfibrils would be enhanced by the putative ability of the cellulose-like domains of the ß-glucan to hydrogen-bond together in so-called junction zones (Fincher and Stone 1986). No significant structural differences in ß-glucan were detected between the two cultivars. This is in agreement with the results obtained by HPLC analysis of oligosaccharides in lichenase digests of whole grain samples.

Chemical fractionation data and electron microscopy have indicated that in barley and wheat the endosperm cell walls are layered: an outer layer consisting of an insoluble polysaccharide skeleton plus matrix polysaccharides, and a larger inner layer of soluble polysaccharides (Mares and Stone 1973a; Fincher 1975; Palmer 1975). While no electron microscopic studies have been performed on the oat endosperm cell walls isolated in this study, the solubility behaviour of the walls is consistent with the results from wheat and

barley. The large proportion of easily solubilized polysaccharide (mostly ß-glucan) extracted from oat endosperm cell walls at 65° would correspond to the inner layer of arabinoxylan and arabinoxylan-plus-ß-glucan that have been suggested to make up the inner layer of the walls of wheat and barley respectively (Mares and Stone 1973a; Fincher 1975; Palmer 1975). The presence of an inner, more easily solubilized layer consisting mostly of ß-glucan suggests that, in addition to the possible transport and/or protective functions discussed in Section 3, ß-glucan may play a storage role in the seed. While digestion of the cell wall during germination is necessary for the intracellular protein and starch to become accessible, a significant amount of glucose would also be liberated, and thus be available for the growing embryo. Support for a storage role for oat ß-glucan is found in studies of cell wall metabolism in oat coleoptiles. When oat coleoptiles were grown in the dark without an energy source, cell wall ß-glucan was catabolized, suggesting a function as a reserve polysaccharide (Loescher and Nevins 1972; Nevins *et al* 1977).

The hot water residue of the endosperm cell wall would correspond to the microfibrillar skeleton (cellulose and glucomannan) plus matrix polysaccharides (ß-glucan and arabinoxylan) that make up the putative outer layer of the wall. Indirect evidence for the presence of arabinoxylans in the outer layer of the cell wall is found in the micrographs showing autofluorescence of phenolics in the endosperm (Fig. 23a,b), which are known to be linked to the arabinoxylans in the cell walls of the Gramineae (Geissmann and Neukom 1973; Smith and Hartley 1983; Hartley *et al* 1990).

5. GENERAL DISCUSSION

Reports in the literature have indicated that there is variation in the ß-glucan content of different cultivars of oats and barley, and that environment may also influence ß-glucan levels in these cereals (Aastrup 1979; Coles 1979; Gill *et al* 1982; Molina-Cano and Conde 1982; Hesselman and Thomke 1982; Bourne and Wheeler 1984; Åman 1986; Henry 1986; Palmer and MacKenzie 1986; Lehtonen and Aikasalo 1987; Truelsen 1987; Åman and Graham 1987a,b; Welch and Lloyd 1989; Welch *et al* 1991; Wood *et al* 1991a). Although barley has been relatively well studied, information concerning ß-glucan variation in oats is somewhat limited. Based on the information available, however, it was hypothesized that the ß-glucan content of oat germplasm varied based on the genotype of the oats, and on the environmental conditions of growth.

Genetic and environmental contributions to variation in total ß-glucan content in Canadian oats were assessed in several cultivars grown in 5 locations in Eastern Canada over 3 years. Although the environmental component was significant in all 3 years of the study, the predominant influence on ß-glucan concentration in oats was clearly genotype. In an attempt to elucidate some of the environmental influences, the relative effects of precipitation and temperature were examined more closely for 4 cultivars which were grown in all locations for all years of the study. Results indicated a low but significant negative association between ß-glucan content and precipitation ($r=-.40$), and an even weaker (but still significant) positive association between temperature and ß-glucan content ($r=-.26$). When species outside of the domestic A. sativa were included in the study, the range in ß-glucan content was found to be somewhat broader than in the domestic cultivars alone. This broader range was mostly due to low ß-glucan contents (less than 3%) in some of the primitive species. No relationship was found between ß-

glucan content and three of the major (and more traditional) quality parameters in oats: groat protein content, oil content and thousand kernel weight. The independent nature of these characteristics emphasizes some of the obstacles facing breeders wishing to improve ß-glucan content in oats: while it is relatively simple to select for a single characteristic, it becomes much more difficult to achieve adequate levels of multiple characteristics in a single cultivar (Newman and Newman 1991).

Variation in ß-glucan was not restricted to variation in total quantities in different cultivars. Differences in the molecular structure of oat ß-glucans, as indicated by differences in the ratio of trisaccharide:tetrasaccharide after lichenase digestion were also observed among different cultivars of Avena sativa and among the primitive species of Avena as well. In addition, scanning microspectrofluorometry showed that the distribution of ß-glucan in single kernels of oats was not uniform. In all of the cultivars examined, the largest deposition was observed in the proximal portion of the kernel, immediately adjacent to the germ. High concentrations were also observed around the periphery of the kernel, in the sub-aleurone layer. This pattern was most noticeable in the cultivars with lower ß-glucan levels. As the total ß-glucan content of the cultivars increased, the mid-endosperm concentration increased also, to the point where in the cultivar with the highest ß-glucan content, there was no clear distinction between the sub-aleurone layer and mid-endosperm with respect to ß-glucan distribution. Although examination of the distribution of ß-glucan within the groat is limited to only a few cultivars at this time, the potential for selection of material to produce brans enriched in ß-glucan content is obvious.

The distribution patterns observed suggest that this cell wall polysaccharide plays more than a structural role in the oat kernel. The high water-holding capacity of ß-

glucans coupled with localized high concentrations adjacent to the germ and around the periphery of the kernel suggest that after imbibition, the germinating oat could survive periods of drought by virtue of the moisture retained by the ß-glucans. In addition, the hydrated ß-glucan in the cell walls could form a sort of "solute highway" during germination between the nutrient-rich endosperm and the growing embryo. Although starch is the major storage carbohydrate in the oat, as germination progresses, hydrolysis of endosperm cell wall ß-glucan could provide an additional source of glucose for the emerging plant.

In the oat caryopsis, the main source of ß-glucan is the endosperm cell wall, and it was hypothesized that the composition of the endosperm cell walls would vary with the total ß-glucan content of the cultivar from which the walls were isolated. To determine whether this was, in fact, the case, endosperm cell walls were isolated from two domestic cultivars identified as containing high and low levels of ß-glucan (Marion and OA516-2, respectively). Isolation of oat endosperm cell walls represented a considerable obstacle, requiring extensive modification of an existing protocol for isolation of wheat endosperm cell walls (Mares and Stone 1973a). In both cultivars, ß-glucan was demonstrated to be the major cell wall polysaccharide in the oat endosperm, constituting up to 85% of the cell wall. Although significant differences were observed in the ß-glucan content of oats (determined on a groat basis), these differences were not reflected in the composition of endosperm cell walls. No significant differences were observed in ß-glucan content, monosaccharide composition, or fractionation characteristics between the two types of cell walls. Microspectrofluorometric analysis and microscopic examination of sections from both cultivars suggested that the differences in ß-glucan contents were due, at least in part, to differences in cell wall layout in the kernel.

A potential model of the oat endosperm cell wall: The preliminary characterization of the oat endosperm cell wall suggests a wall structure that is similar to that which has been proposed for the endosperm cell wall of barley (Fincher 1975) Although both cereals contain similar levels of ß-glucan, the higher solubility observed in oat endosperm cell walls relative tc those of barley (Ballance and Manners 1978) suggests that oat products may be more useful adjuncts in the management of mild hypercholesterolemia and diabetes.

The solubility characteristics of isolated oat endosperm cell walls are consistent with a layered model: a relatively thin outer layer (adjacent to the middle lamella) consisting of an insoluble polysaccharide skeleton plus matrix polysaccharides, and a large inner layer of soluble polysaccharides. These layers correspond to the residue after hot (65°C) water extraction, and the hot water extract, respectively. A diagrammatic representation of the proposed model of the oat endosperm cell wall is presented in Fig. 29.

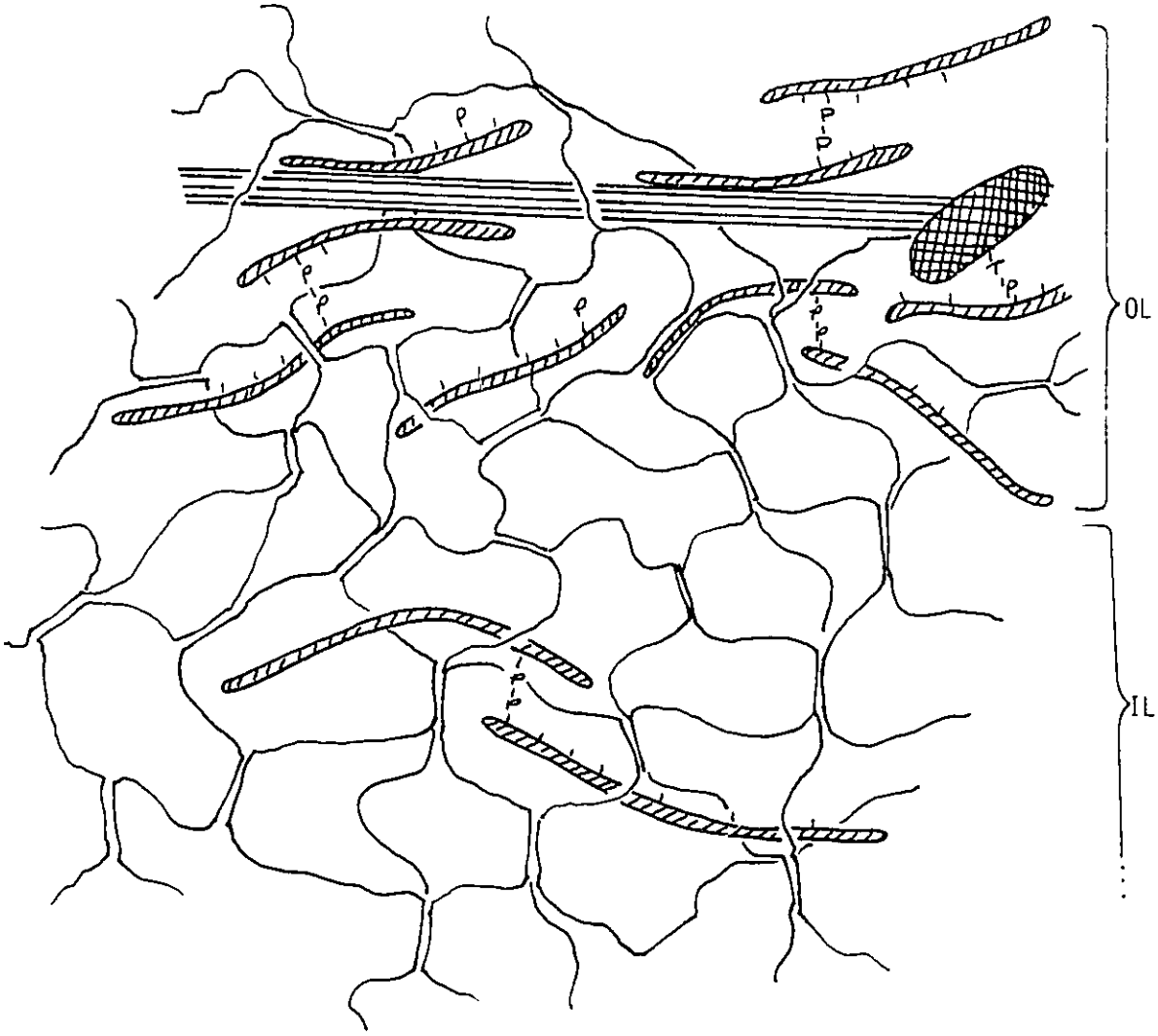
In the proposed model (Fig. 29), the insoluble skeleton consists of microfibrils of cellulose and glucomannan. Surrounding this skeleton is a matrix of ß-glucan and arabinoxylan. ß-Glucan molecules are connected to the insoluble skeleton, and also to each other by hydrogen bonding along the cellulose-like surfaces presented by stretches of consecutive (1→4) linkages (junction zones: Fincher and Stone 1986). Arabinoxylan molecules could also bind to the skeleton by hydrogen bonds in regions of the molecule with low degrees of substitution, as well as to cellulose-like regions of ß-glucan molecules. Phenolic acids (Fig. 29, P) are attached to arabinose sidechains on the arabinoxylans (Geissmann and Neukom 1973; Smith and Hartley 1983; Hartley *et al* 1990), and the arabinoxylans are probably crosslinked by the formation of diphenolic

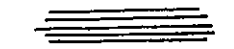
bridges (Ishii 1991), which would contribute to the insolubility of the outer layer. A higher proportion of arabinoxylan is shown in the outer layer in Fig. 29 because analysis of the hot water residue (Section 4.3.2.6) showed that it was enriched in arabinoxylan relative to the whole cell wall. Microscopic evidence showing autofluorescence at the outside of the cell wall (Fig. 23) also indicates the presence of arabinoxylans in the outer part of the wall.

Information on the protein component of the oat endosperm cell wall is at present limited to amino acid composition, which provides no information about structure, or even the number of proteins present, so the representation of protein in this model is speculative at best. The shape used to represent protein is arbitrary, and is intended only to indicate the presence and relative position in the cell wall. The protein is probably associated with the insoluble skeleton, or at least in the outer layer of the wall, because removal of a large portion of the cell wall by lichenase digestion did not solubilize detectable amounts of protein. The possibility of bridge formation between tyrosine residues in protein, and ferulic acid residues on the arabinoxylans has been raised by other workers (Neukom and Markwalder 1978), and if these were also present, it would help account for the insolubility of the protein. The amino acid composition determined on the oat endosperm cell wall preparation did not have the high proportion of hydroxyproline and serine that is characteristic of cell wall structural proteins (Wilson and Fry 1986), so the protein may not be structural in nature, but possibly an enzyme or an enzyme complex.

Hot water extraction data indicates that the inner layer of the cell wall is mostly ß-glucan, with a small amount of arabinoxylan. The proportion of inner and outer layer shown in Fig. 28 is not intended as an accurate representation of the proportions of these

Figure 29. Diagram showing a model of the oat endosperm cell wall. OL, outer layer; IL, inner layer; P, phenolic group; P-P, diphenolic cross-link; T-P, putative tyrosine-phenolic crosslink.

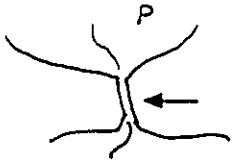


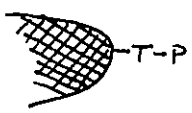

insoluble skeleton

arabinoxylan

phenolic group

phenolic crosslink


β-glucan gel-matrix with junction zone (arrow)



protein with possible tyrosine-phenolic (T-P) crosslink

layers in the wall: the diagram is primarily meant to show the proposed relationships of the cell wall components. In the proposed model, the ß-glucan in the inner layer of the wall forms a sort of gel-matrix by forming junction zones between stretches of (1→4)-linked glucosyl residues. The arabinoxylan present would be held in the wall by the formation of diphenolic bridges, and also by entanglement with the ß-glucan molecules. Junction zones between areas with low degrees of substitution, and cellulose-like regions of ß-glucan are also a possibility.

The results reported in this thesis offer an improved definition of oat ß-glucan,in terms of the whole oat, and in the endosperm cell wall as well. Variations in content and distribution of ß-glucan that have been observed in oats provide deeper insight into the nature of this unique polysaccharide, as well as offering new perspectives on ways to exploit the reported benefits of oat products as dietary adjuncts for the improvement of lipid and glucose metabolism. To date, a broad survey of the ß-glucan content of Canadian domestic cultivars has not been available, and information concerning the ß-glucan content of primitive species of Avena has been limited as well. Information on the composition of the endosperm cell wall, which is the major source of ß-glucan in oats, was similarly lacking. With the current interest in the clinical benefits of oat ß-glucan in human nutrition on one hand, balanced by the detrimental effects reported in livestock feeding trials on the other, this type of information will allow a choice of specific cultivars to maximize the desired effect.

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