

# Carbohydrate-Degrading Enzymes in Germinating Wheat

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## ABSTRACT

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The production of carbohydrate-degrading enzymes was followed during the first five days of germination of wheat (cultivar Hartog).  $\alpha$ -Amylase (EC 3.2.1.1) increased from the first day to reach a peak after four days.  $\beta$ -(1 $\rightarrow$ 3)(1 $\rightarrow$ 4)-Glucanase (EC 3.2.1.73) increased from day one to day five. Endo-(1 $\rightarrow$ 4)- $\beta$ -xylanase (EC 3.2.1.8) activity increased only slowly until the fifth day when activity increased more than threefold.  $\beta$ -Fructofuranosidase (EC 3.2.1.26) was not detected until the third day. Movement of hydrolytic enzymes into the endosperm and thus milling

fractions may be controlled by enzymes degrading the cell walls. Staining with fluorescein dibutyrate indicated that on average more than 30% of the endosperm had been penetrated by lipase-esterase activity by the fifth day. All activities declined when the grain was dried at 30°C but the effect of drying varied.  $\alpha$ -Amylase activity was reduced by 69%, whereas  $\beta$ -amylase (EC 3.2.1.2) activity declined by only 16%. Residual activities of hydrolytic enzymes in sprouted wheat may be determined by environmental conditions during grain drying.

Preharvest sprouting of wheat results in the production of a range of hydrolytic enzymes in the germinating grain. The loss in quality of sprouted wheat has been largely attributed to the presence of these enzymes (Kruger 1976).

Most research has focused on  $\alpha$ -amylase and the effects of hydrolysis of starch in the grain and in doughs. Other flour constituents such as protein and cell wall polysaccharides (for example, pentosans) are important in establishing quality for many end uses. Hydrolysis of these components in sprouted wheat products may also contribute to loss of quality. Enzymes hydrolyzing endosperm cell walls have not been well characterized in wheat (Kruger and Lineback 1987).

This paper compares the rates of production of  $\alpha$ -amylase,  $\beta$ -amylase invertase, and a range of cell wall (pentosan and  $\beta$ -glucan) degrading enzymes during the first five days of germination. Since sprouted wheat is usually subjected to a period of drying in the field or after harvest, the effect of drying on enzyme activities in wheat after three days of germination was also investigated.

## MATERIALS AND METHODS

### Sources of Substrates

Sucrose, cellobiose, laminaribiose, xylobiose, carboxymethyl-cellulose, laminarin, xylan (oat), 4-O-methyl-D-glucurono-D-xylan-Remazol brilliant blue R, *p*-nitrophenyl  $\beta$ -D-xylopyranoside, *p*-nitrophenyl  $\alpha$ -L-arabinopyranoside, and *p*-nitrophenyl  $\alpha$ -L-arabinofuranoside were obtained from Sigma Chemical Company. Barley (1 $\rightarrow$ 3)(1 $\rightarrow$ 4)- $\beta$ -glucan and azo-barley  $\beta$ -glucan were from Biocon Biochemicals. Cross-linked Cibachron Blue-dyed starch tablets (Barnes and Blakeney 1974) were from Pharmacia. Arabinoxylan was isolated from rye using a method similar to that described by Gibson and McCleary (1987).

### Isolation of Rye Arabinoxylan

The endogenous enzymes in 200 g of ground rye (*Secale cereale* cv. South Australian) were inactivated by boiling in 1.5 L of 80% v/v ethanol for 10 min. The pellet collected by centrifugation at 3,000  $\times$  g for 10 min was suspended in 700 ml of distilled water and heated at 40°C for 1 hr with stirring. The supernatant was collected by centrifugation at 3,000  $\times$  g for 30 min and the pellet extracted similarly with a further 600 ml of distilled water. The combined supernatants were adjusted to pH 5.0 by adding concentrated sodium acetate buffer (final concentration, 50 mM) and calcium chloride to give a final 1 mM concentration. The solution was heated to 100°C and 2 ml of a heat-stable  $\alpha$ -amylase (EC 3.2.1.1, Thermamyl, Novo) was added to digest starch. The mixture was incubated at 100°C for 30 min and then cooled to room temperature. Arabinoxylans were precipitated overnight at 40°C by adding three volumes of ethanol. The pellet recovered by centrifugation at 3,000  $\times$  g for 10 min was resuspended in 1 L of 0.5M maleate buffer (pH 6.5), and 20 ml of (1 $\rightarrow$ 3)(1 $\rightarrow$ 4)- $\beta$ -glucanase (EC 3.2.1.3) (Henry and Blakeney 1988) was added and incubated at 40°C for 2 hr to hydrolyze  $\beta$ -glucans. The arabinoxylan was precipitated overnight at 4°C by adding three volumes of ethanol. The pellet recovered by centrifugation at 3,000  $\times$  g for 10 min was dried under vacuum and ground finely using an Ultramat dental amalgamator. Acid hydrolysis and analysis of monosaccharides by gas chromatography as their alditol acetates (Blakeney et al 1983) indicated that the monosaccharides in this preparation were 64% xylose and 36% arabinose with no detectable glucose. No other monosaccharides were detected. Solutions of this preparation had a viscosity similar to those of (1 $\rightarrow$ 3)(1 $\rightarrow$ 4)- $\beta$ -glucan from barley (Biocon).

The rye arabinoxylan was dyed with Remazol brilliant blue R as described by Biely et al (1985).

### Germination of Wheat

Wheat (*Triticum aestivum* cv. Hartog) was grown in the field at Jimbour in Queensland in 1987. Seed was surface sterilized by soaking in 1% sodium hypochlorite in distilled water for 20 min and then washed with distilled water. Germination was at 16°C in a Seeger micromalting plant. The seed was irrigated with a fine mist of water from above for 10 sec every 30 min and

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turned over by hand every 24 hr. These germination conditions allowed maximum water uptake without restricting oxygen supply as may occur during steeping. Germination was stopped after three days by rapidly freezing the samples in liquid nitrogen followed by freeze-drying or by drying in a stream of air for 24 hr at 30°C in the kiln of the micromalting plant. In a separate experiment, samples were frozen after one, two, three, four, and five days of germination to establish the pattern of enzyme production during germination. All samples were extracted in duplicate and assayed for enzyme activity.

#### Extraction of Enzymes

The dry seed was ground to pass a 0.8-mm sieve using a laboratory hammer mill (falling number 3,100). Enzymes were extracted by adding 5 ml of 50 mM sodium maleate buffer (pH 5.2) containing 2 mM calcium chloride, 50 mM sodium chloride, and 3 mM sodium azide to 0.500 g of the ground sample and agitating several times with a vortex mixer. Extracts were centrifuged at 1,000 × *g* for 10 min after extraction for 10 min at room temperature.

#### α-Amylase Assay

α-Amylase (EC 3.2.1.1) was assayed as described previously using insoluble dye-labeled starch as substrate (Henry 1985). Enzyme units are as described by Barnes and Blakeney (1974).

#### β-Amylase Assay

β-Amylase was assayed by the method of McCleary and Codd (1989) using *p*-nitrophenyl-α-D-maltopentose as substrate and incorporating cysteine in the extraction buffer to extract the "bound" enzyme from ungerminated grain.

#### Endo-β-Xylanase Assay

Endo-β-xylanase (EC 3.2.1.8) activity was assayed by adding 0.5 ml of extract to 0.5 ml of 11.5 mg/ml 4-O-methyl-D-glucuron-D-xylan-remazol brilliant blue R (Biely et al 1985) in 0.1M sodium acetate buffer (pH 5.4) containing 10 mg/ml bovine serum albumin (Sigma) and incubating at 30°C for 60 min. The reaction was stopped by adding 2 ml of ethanol and mixing. The absorbance of the supernatant at 595 nm was recorded after centrifugation at 2000 × *g* for 10 min. The reaction was found to be linear with time over 1 hr under these conditions.

#### Endo-β-Glucanase Assay

Endo-(1→3)(1→4)-β-glucanase was assayed using azo-barley β-glucan as substrate as described by McCleary and Shameer (1987). One enzyme unit is the amount of activity that cleaves one

micromole of glycosidic linkages per minute under the conditions defined by McCleary and Shameer (1987).

#### Assay of Glycosidases with *p*-Nitrophenyl-Linked Substrates

These enzymes were assayed by incubating 0.2 ml of the extracted enzyme with 0.2 ml of 2.5 mg/ml *p*-nitrophenyl-glycoside in 50 mM sodium acetate buffer (pH 5.0) at 40°C for 10 min. The reaction was stopped by adding 3.0 ml of 1%, w/v, tris (hydroxymethyl) aminomethane. The absorbance was recorded at 400 nm.

#### Other Carbohydrate-Degrading Enzymes

Other carbohydrate-degrading enzymes were assayed by incubating 0.5 ml of enzyme with 0.5 ml of 2.5 mg/ml substrate in 50 mM sodium acetate buffer pH 5.0 at 40°C for up to 1 hr. The reaction was stopped by adding 1.0 ml of 0.5M sodium hydroxide. Reducing sugars were determined by reaction with *p*-hydroxybenzoic acid hydrazide (Blakeney and Mutton 1980). Glucose was determined where appropriate, using glucose oxidase (Blakeney and Matheson 1984). The viscosity of assay mixtures involving arabinoxylan from rye as substrate was also monitored with a U-tube viscometer.

#### Fluorescein Dibutyrate Staining

Wheat was embedded, stained with fluorescein dibutyrate, and examined by fluorescence microscopy as previously described (Henry and McLean 1986).

## RESULTS AND DISCUSSION

#### Effect of Drying on Enzyme Activities in Germinated Wheat

The activity of all hydrolytic enzymes examined was lower in air-dried wheat (30°C) than in freeze-dried samples (Table I). The greatest reduction in activity (69%) was observed for α-amylase, whereas β-amylase was apparently much more stable to drying (84% retained after drying), and some glycosidases retained 79% of activity. However, the lower reduction in activity of some of these enzymes following air-drying may reflect an increase in the production of these enzymes during the early stages of drying (except for β-amylase, which is not produced during germination) rather than greater heat stability of the enzymes. α-Amylase synthesis may be more sensitive to higher temperatures (Henry and McLean 1987) than the synthesis of some other enzymes. Higher temperatures during drying may initially accelerate the production of some enzymes until moisture or temperature becomes limiting, thus reducing the apparent loss of activity. A further specific cause for the larger reduction in

TABLE I  
Effect of Drying on the Activity of Enzymes in Germinating Wheat (all assays were on wheat germinated for three days)

Enzyme	Substrate	Activity		Loss of Activity on Air-Drying <sup>a</sup> (%)
		Air-Dried	Freeze-Dried	
α-Amylase (EC 3.2.1.1)	starch (dyed and crosslinked)	12.6 U/g	40.6 U/g	69
β-Fructofuranosidase (EC 3.2.1.26)	sucrose	322 <sup>b</sup>	743 <sup>b</sup>	57
β-Xylanase (EC 3.2.1.8)	4-O-methyl glucurono xylan RBB	8 <sup>c</sup>	18 <sup>c</sup>	56
β-Glucanase (EC 3.2.1.73)	azo (1→3)(1→4)-β-glucan barley	65.7 U/kg	93.4 U/kg	30
β-D-Xylopyranosidase	<i>p</i> -nitrophenyl-β-D-xylanopyranoside	0.22 <sup>d</sup>	0.28 <sup>d</sup>	21
α-L-Arabinopyranosidase	<i>p</i> -nitrophenyl-α-L-arabinopyranoside	0.23 <sup>d</sup>	0.29 <sup>d</sup>	21
α-L-Arabinofuranosidase	<i>p</i> -nitrophenyl-α-L-arabinofuranoside	0.16 <sup>d</sup>	0.20 <sup>d</sup>	20
β-Amylase (EC 3.2.1.2)	<i>p</i> -nitrophenyl-α-D-maltopentose	718 U/g	856 U/g	16

<sup>a</sup>Percentage of activity in freeze-dried grain lost on air-drying.

<sup>b</sup>Glucose equivalents (μmol)/min/kg.

<sup>c</sup>Arbitrary units.

<sup>d</sup>A<sub>400</sub> increase in standard assay.

$\alpha$ -amylase activity may be the production of  $\alpha$ -amylase inhibitors during drying. Mundy (1984) showed that the barley  $\alpha$ -amylase/subtilisin inhibitor is produced in response to abscisic acid. The wheat  $\alpha$ -amylase/subtilisin inhibitor (Mundy et al 1984) may be produced in response to abscisic acid produced during grain drying (Wright and Hiron 1969). These results suggest that the drying conditions in the field are likely to be very important in determining the relative levels of hydrolytic enzymes in wheat that has been subject to preharvest sprouting.

#### $\alpha$ -Amylase

The production of  $\alpha$ -amylase in germinating cereals has been studied extensively. However, the results presented here provide a reference for comparison of the rates of production of other enzymes.

$\alpha$ -Amylase activity increased over the first four days but showed little increase on the fifth day of germination (Fig. 1). These results are consistent with results of previous studies (Duffus 1987). After an initial lag,  $\alpha$ -amylase levels rise quickly and reach a maximum after a few days (Atzorn and Weiler 1983).

#### $\beta$ -Amylase

Most of the  $\beta$ -amylase released during germination is present in an insoluble or latent form in the ungerminated grain (Kruger 1970). Total  $\beta$ -amylase extracted with cysteine increased only slightly during germination. The ungerminated grain contained 907 U/g of  $\beta$ -amylase, which increased to 1,030 U/g over four days (Fig. 1). This very small apparent increase may be due to increasing ease of extraction since  $\beta$ -amylase is not produced during germination.

#### Endo-Xylanase

The major component of endosperm cell walls from wheat is arabinoxylan (Mares and Stone 1973). Endo-(1 $\rightarrow$ 4)- $\beta$ -xylanase was very low over the first three days but increased rapidly on the fourth and fifth days (Fig. 2). This appears to be the first report of the assay of cereal xylanases with 4-O-methyl-D-glucurono-D-xylan-Remazol brilliant blue R as substrate. The significance of the delayed production of this enzyme and its location within the grain have not been established.

The late appearance of this pentosan-degrading enzyme is also supported by the finding of Schmitz et al (1974) that steeping

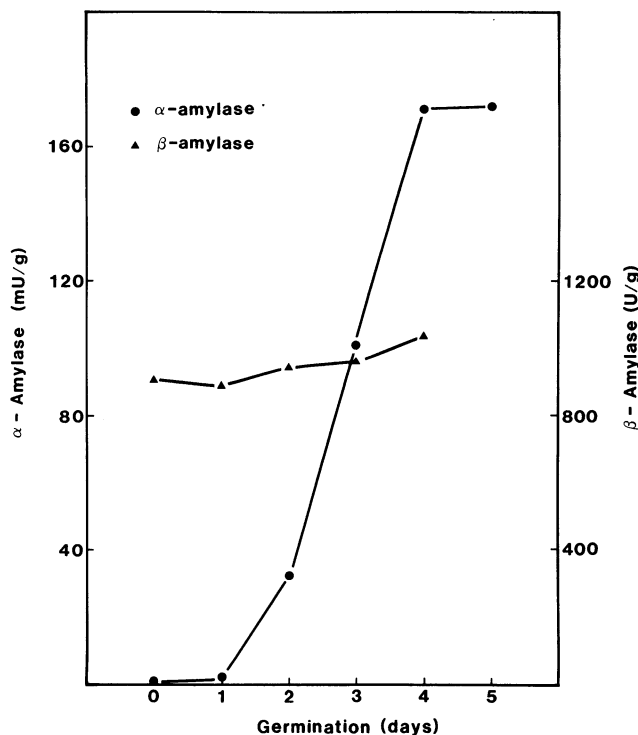


Fig. 1.  $\alpha$ -Amylase and  $\beta$ -amylase activity in germinating wheat.

of wheat decreased the falling number but did not increase endo-pentosanase activity measured viscometrically. Hydrolysis of xylobiose or arabinoxylans from oat and rye by enzymes in extracts of germinating wheat was not detectable by a reducing sugar assay in the present study. However, extracts from wheat germinated for five days reduced the viscosity of arabinoxylan from rye and hydrolyzed arabinoxylan dyed with Remazol brilliant blue.

The addition of bovine serum albumin to the assay mixture reduced blank absorbance values, presumably by binding and precipitating free dye molecules. The higher protein concentration may also help to stabilize the xylanase.

#### $\beta$ -Glucanases

Wheat endosperm cell walls also contain (1 $\rightarrow$ 3)(1 $\rightarrow$ 4)- $\beta$ -glucans (Bacic and Stone 1980). The activity of endo-(1 $\rightarrow$ 3)(1 $\rightarrow$ 4)- $\beta$ -glucanases increased throughout the five days of germination (Fig. 2). Stuart et al (1987) reported (1 $\rightarrow$ 3)(1 $\rightarrow$ 4)- $\beta$ -glucanase and (1 $\rightarrow$ 3)- $\beta$ -glucanase but little (1 $\rightarrow$ 4)- $\beta$ -glucanase activity in germinating wheat and other cereals. Enzymes capable of reducing the viscosity of carboxymethyl cellulose have been reported in flour fractions (Schmitz et al 1974), but only traces of activity on cellobiose were found. In the present study, activities of enzymes hydrolyzing laminarin and barley (1 $\rightarrow$ 3)(1 $\rightarrow$ 4)- $\beta$ -glucan were present. Carboxymethyl cellulose, laminaribiose, and cellobiose were not hydrolyzed to produce significant amounts of reducing sugars. There is no evidence that endo-(1 $\rightarrow$ 3)(1 $\rightarrow$ 4)- $\beta$ -glucanase is produced before  $\alpha$ -amylase as has been reported for barley (MacLeod et al 1964).

#### Glycosidases

The activity of enzymes hydrolyzing *p*-nitrophenyl glycosides of arabinose and xylose increased during germination (Fig. 3). Lee and Ronalds (1972) reported low levels of  $\alpha$ -arabinosidase and  $\beta$ -xylosidase in flours from sound wheat and suggested that they may represent secondary activities of  $\beta$ -galactosidases and  $\beta$ -glucosidases. Arabinopyranosidase and xylosidase had

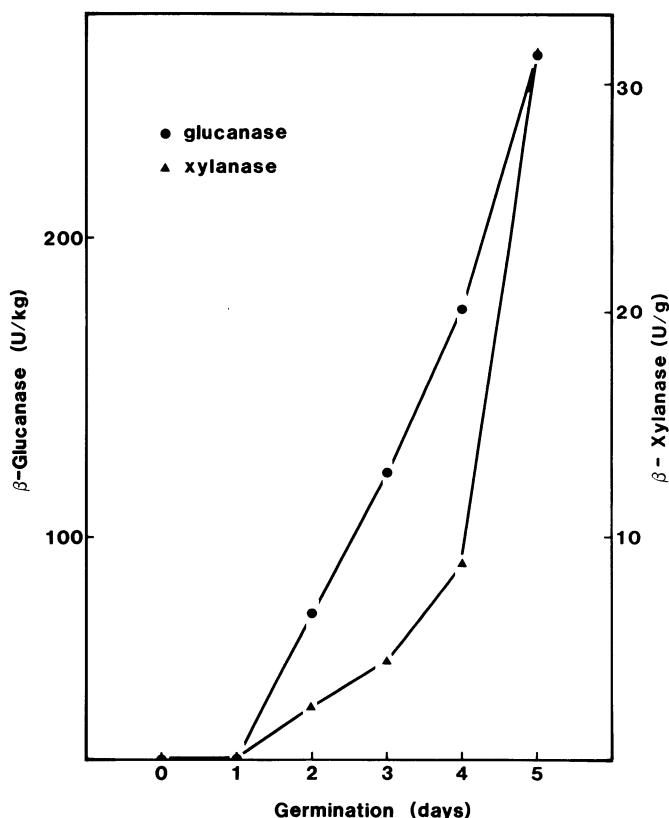


Fig. 2. Activity of (1 $\rightarrow$ 3)(1 $\rightarrow$ 4)- $\beta$ -glucanase and endo- $\beta$ -xylanase in germinating wheat (xylanase activity unit = change in absorbance per minute per gram of wheat).

significant activities in the sound grain before germination in the present study, whereas most other enzymes were not detectable. Schmitz et al (1974) found no increase in xylosidase activity on steeping, whereas in the present study xylosidase activity was found to increase more rapidly than arabinosidase activity throughout germination.

### Invertase

Invertase ( $\beta$ -fructofuranosidase, EC 3.2.1.26) activity was very low until the third day but continued to increase on the fourth and fifth days (Fig. 4).

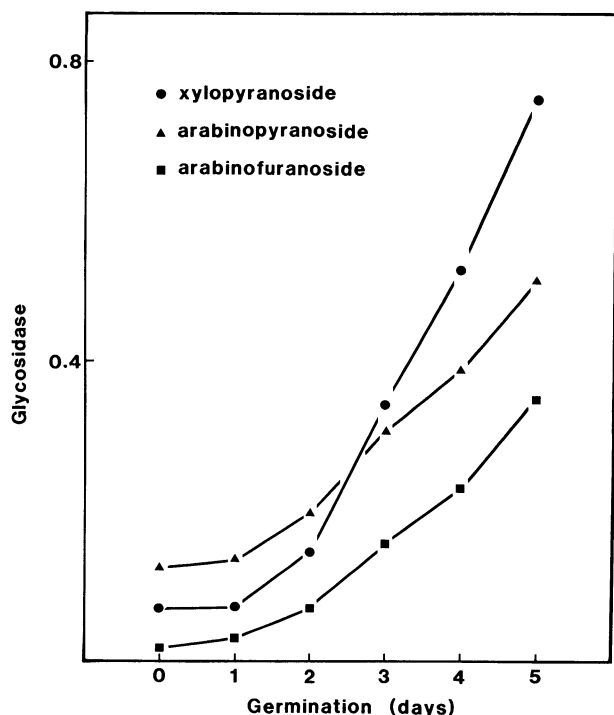


Fig. 3. Hydrolysis of *p*-nitrophenyl-glycosides by enzymes from germinating wheat ( $A_{400}$  increase in standard assay used as arbitrary activity units).

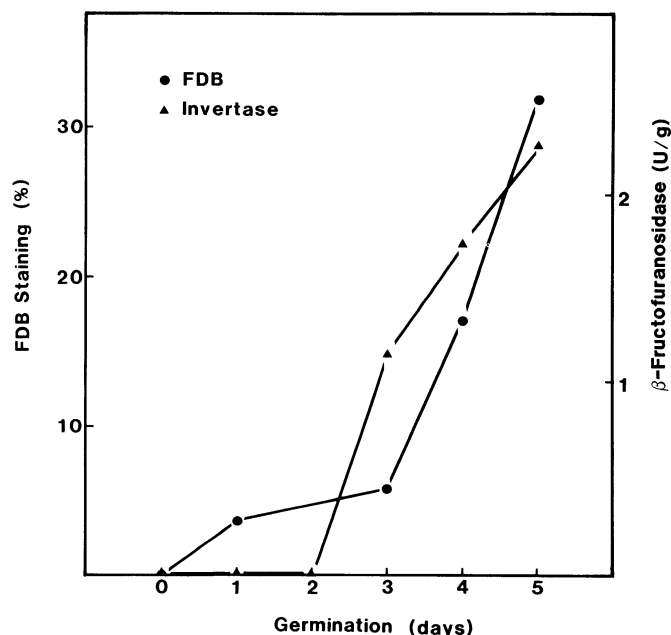


Fig. 4.  $\beta$ -Fructofuranosidase (invertase) activity in germinating wheat, expressed as micromoles (glucose equivalents) per minute per gram of wheat, and fluorescein dibutyrate staining of germinating wheat. The mean percentage of the endosperm stained for 50 grains is plotted against the time of germination.

Invertase declines to low levels as the wheat grain reaches maturity and is mainly in an insoluble form located in the pericarp (Chevalier and Lingle 1983). The sucrose content of barley embryos declines rapidly in the first 24 hr of germination (James 1940), but the sucrose content of the whole grain increases in the later stages of malting (Harris and MacWilliam 1954). The increase in soluble invertase measured in the present study may be associated with a similar later increase in sucrose concentrations.

### Penetration of Endosperm by Hydrolytic Enzymes

The penetration of lipase/esterases into the endosperm as indicated by the portion of the endosperm stained by fluorescein dibutyrate is shown in Figure 4. This process is relatively slow over the first few days of germination but is increasing rapidly by the fifth day. The rate of movement of other enzymes such as  $\alpha$ -amylase, produced in large quantities early in germination, may be limited by the availability of endo-xylanase to degrade endosperm cell walls composed largely of arabinoxylan.

### Significance for the Utilization of Sprouted Wheat

The amounts of hydrolytic enzymes, especially  $\alpha$ -amylase, and their distribution within the endosperm are both important factors that may limit the utilization of sprouted wheat. Environmental conditions during grain drying (e.g., temperature, humidity, and wind speed) may determine the relative levels of residual enzyme activity in the dry grain. Enzymes such as endo-xylanases may influence the quality of the flour recoverable from sprouted wheat by determining the extent to which  $\alpha$ -amylase penetrates into the endosperm. These considerations may be important in attempts to upgrade damaged wheat by improving processing techniques (Henry et al 1987) and in the breeding of wheats with genetic resistance to sprouting (Henry and Brennan 1988).

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