



Influence of waxy and high-amylose starch genes on the composition of barley and the cholesterolemic and glycemic responses in chicks and rats  
by Qi Xue

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in  
Crop and Soil Science  
Montana State University  
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**Abstract:**

The ratio of amylose vs amylopectin (waxy) starch in barley grain affects its chemical composition and biological properties. To investigate the effects of waxy (ww) and high-amylose (amol) starch genes on chemical composition of barley and the cholesterolemic and glycemic responses in an animal model, a series of barley isotypes having non-waxy, waxy starch and another series of Glacier barley having high-amylose (HA) starch in covered, and hull-less lines were tested. Waxy gene increased ( $P < .01$ ) total and soluble dietary fiber, total and soluble  $\beta$ -glucans, extract viscosity, free sugars, and reduced starch content of barley. Extract viscosity was correlated with total  $\beta$ -glucan and free sugars ( $r = .55$  and  $.76$ ,  $P < .001$ ). High-amylose gene increased amylose content (40%-44%) and  $\beta$ -glucan content (7.4%) over normal barley (29%, 5.2%). Means of postprandial blood glucose levels were not different for chicks fed uncooked (raw) high-amylose barley flour or red dog (RD) milling fractions compared with normal barley but were higher ( $P < .05$ ) compared with com controls. However, total and LDL plasma cholesterol were lower for chicks fed the HA flours and all barley RD diets compared to com controls. Starches were purified from barley flours of waxy, normal, and high-amylose cultivars. These starches were moisture-autoclaved 3 and 12 times with subsequent cooling. Only marginal effects occurred on hydrolysis rates with no effects on glucose levels in rats when boiled starches of different origins (amylose vs amylopectin) were tested. However, the effects of starch types on digestibility and blood glucose responses were significantly altered by autoclaving. Digestibility of waxy starches was not changed in vitro ( $P > .05$ ) but glucose in rats was significantly ( $P < .05$ ) increased after ingestion of autoclaved waxy barley starch. In contrast, the digestibilities of high-amylose starches were reduced by 14% and 20% after 3 and 12 autoclave-cool cycles, respectively. Autoclaved HA starch significantly lowered the glucose peaks in rats compared to waxy and normal starches at 30 min ( $P < .01$ ). The in vivo results corresponded to that in vitro study which demonstrated that the digestibility of different cereal starches followed the pattern: waxy > normal > high-amylose starches after heat-moisture autoclaving, possibly due to the formation of enzyme resistant starch from the amylose component. Enzyme resistant starch formation was highly ( $P < .001$ ) correlated with digestibility ( $r = -.98$ ) and amylose contents ( $r = .96$ ) of autoclaved starches. These data indicate that the expression of the waxy and high-amylose genes in barley is desirable to increase the levels of soluble dietary fiber, particularly  $\beta$ -glucans and also implied that high amylose barley has potential for specialty food product development.

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## ABSTRACT

The ratio of amylose vs amylopectin (waxy) starch in barley grain affects its chemical composition and biological properties. To investigate the effects of waxy (*ww*) and high-amylose (*amo1*) starch genes on chemical composition of barley and the cholesterolemic and glycemic responses in an animal model, a series of barley isotypes having non-waxy, waxy starch and another series of Glacier barley having high-amylose (HA) starch in covered, and hull-less lines were tested. Waxy gene increased ( $P < .01$ ) total and soluble dietary fiber, total and soluble  $\beta$ -glucans, extract viscosity, free sugars, and reduced starch content of barley. Extract viscosity was correlated with total  $\beta$ -glucan and free sugars ( $r = .55$  and  $.76$ ,  $P < .001$ ). High-amylose gene increased amylose content (40%-44%) and  $\beta$ -glucan content (7.4%) over normal barley (29%, 5.2%). Means of postprandial blood glucose levels were not different for chicks fed uncooked (raw) high-amylose barley flour or red dog (RD) milling fractions compared with normal barley but were higher ( $P < .05$ ) compared with corn controls. However, total and LDL plasma cholesterol were lower for chicks fed the HA flours and all barley RD diets compared to corn controls. Starches were purified from barley flours of waxy, normal, and high-amylose cultivars. These starches were moisture-autoclaved 3 and 12 times with subsequent cooling. Only marginal effects occurred on hydrolysis rates with no effects on glucose levels in rats when boiled starches of different origins (amylose vs amylopectin) were tested. However, the effects of starch types on digestibility and blood glucose responses were significantly altered by autoclaving. Digestibility of waxy starches was not changed *in vitro* ( $P > .05$ ) but glucose in rats was significantly ( $P < .05$ ) increased after ingestion of autoclaved waxy barley starch. In contrast, the digestibilities of high-amylose starches were reduced by 14% and 20% after 3 and 12 autoclave-cool cycles, respectively. Autoclaved HA starch significantly lowered the glucose peaks in rats compared to waxy and normal starches at 30 min ( $P < .01$ ). The *in vivo* results corresponded to that *in vitro* study which demonstrated that the digestibility of different cereal starches followed the pattern: waxy > normal > high-amylose starches after heat-moisture autoclaving, possibly due to the formation of enzyme resistant starch from the amylose component. Enzyme resistant starch formation was highly ( $P < .001$ ) correlated with digestibility ( $r = -.98$ ) and amylose contents ( $r = .96$ ) of autoclaved starches. These data indicate that the expression of the waxy and high-amylose genes in barley is desirable to increase the levels of soluble dietary fiber, particularly  $\beta$ -glucans and also implied that high amylose barley has potential for specialty food product development.

## CHAPTER 1

## INTRODUCTION

Coronary heart disease (CHD) and diabetes mellitus are not only the major causes of death in the industrialized countries, but they account for enormous costs for treatment and care (Anonymous, 1981; NDDG, 1985; Anderson et al., 1987a). Individuals with diabetes die from CHD two to three times more frequently than those who do not have diabetes (Anderson et al., 1987b). Hypercholesterolemia and hyperglycemia were recognized as significant risk factors for CHD (Connor and Connor, 1984) and for diabetes (Anderson et al., 1987b). Reductions in cholesterol and postprandial glucose and insulin in the blood are considered to be beneficial in preventing and treating hyperlipidemia and hyperglycemia as well as related disorders including obesity and hypertension, thereby reducing the risk of CHD and diabetes (Amelsvoort and Weststrate, 1992).

Diets high in fiber and starches have been reported to normalize blood glucose and lipid levels in carbohydrate-sensitive diabetic and hyperlipidemic individuals (Behall and Scholfield, 1989; Jenkins and Jenkins, 1985). Soluble fiber such as  $\beta$ -glucan in barley has been shown to have hypocholesterolemic effects in rats (Klopfenstein and Hosoney, 1987; Mori, 1990), chicks (Fadel et al., 1987), and humans (Newman et al.,

1989a; McIntosh et al., 1991). Bengtsson et al. (1990) concluded that the hypocholesterolemic response observed in a waxy barley, but not in a non-waxy barley was due to the higher level of  $\beta$ -glucan and viscosity in waxy barley. Higher levels of total dietary fiber (TDF),  $\beta$ -glucan and relative extract viscosity have been reported by Ullrich et al. (1986), Mori (1990) and Han and Froseth (1992). Soluble fiber, including  $\beta$ -glucan was demonstrated to flatten glucose curves in humans (Jenkins and Jenkins, 1985) and in rat (Vachon et al., 1988).

The bioavailability of starch, especially the effect of amylose/amylopectin ratio, has recently been an issue of nutritional concern. Research reports on the glucose-lowering response of high-amylose corn starch (Behall and Scholfield, 1989; Amelsvoort and Weststrate, 1992) and rice starch (Goddard et al, 1984; Panlasigui et al, 1992) have been reported. Behall and Scholfield (1989) also reported a lowering of blood triglycerides and cholesterol in subjects consuming high-amylose corn starch.

Few nutritional studies on barley starch have been reported although some barley cultivars are known to have widely varying ratios of amylose to amylopectin. An amylose level of 44% was discovered in a mutant of Glacier. In addition to the starch ratio, high-amylose Glacier barley also contains relatively high  $\beta$ -glucan, indicative of hypocholesterolemic responses.

The objectives of these studies were to (1) determine if increased levels of  $\beta$ -glucans, TDF and extract viscosity in waxy barley are consistent in more than one genotype grown in different environments; (2) evaluate the effects of hull-less and short-awned genes on other nutrient components in addition to the effects of the waxy gene;

(3) investigate glucose and cholesterol responses of broiler chicks fed two uncooked barley milling fractions from three different Glacier barleys; (4) estimate the formation of enzyme resistant starch during the autoclaving process with different ratios of amylose and amylopectin in barley and corn; (5) determine *in vitro* digestibility and hydrolysis rate of barley and corn starches with different amylose and amylopectin content and heat treatments (autoclaved vs non-autoclaved); and (6) evaluate the glucose responses of autoclaved barley starch compared to wheat starch in rats.

## CHAPTER 2

### LITERATURE REVIEW

Barley is an important agricultural crop in the world, and particularly in the Northern Plains States. The grain is used mainly as an animal feed and for malt but there is a growing interest in barley for food and industrial use (Åman and Newman, 1986). A better understanding of the relationship between grain chemical composition and physical characteristics as well as the nutrition value would be helpful in successful utilization of barley for new uses (Henry, 1988). A knowledge of the contribution of genetic and environmental factors for grain composition is also required. Carbohydrates, major components of the kernel, have a significant influence on grain quality. The carbohydrates are generally subdivided into starch, sugar and dietary fiber (DF). This review will focus on aspects of carbohydrate nutrition related to barley starch genotypes.

#### Starch

##### Starch Structure and Composition

Starch makes up about 50 to 68% of barley grain (Åman and Newman, 1986), occurring as water insoluble granules. The shape and size of the granules are markedly different in plants, dependent on the botanical source. For example, normal Glacier

barley starch exhibits bimodal distribution (Banks and Muir, 1980). In these starch granules, there is a population of large lenticular granules, ranging in diameter from 15 - 35  $\mu\text{m}$ , which comprise some 90% by weight but less than 10% by number of the total starch, and a separate population of small spherical granules ranging from 1 to 10  $\mu\text{m}$ . However, high-amylose Glacier barley is quite different from the normal Glacier, having smaller sized granules with less regular shaping and no clear evidence of the bimodal distribution.

Starch granules are semicrystalline and exhibit birefringence in polarized light. Starch granules can give a number of distinct types of X-ray patterns (designated A, B, and C). Information on the organization of the starch granule has been obtained from different microscopy techniques (Lineback, 1984; Zobel, 1992). X-ray diffraction of crystalline amylose helical inclusion compounds results in the V amylose pattern (Hoseney, 1986).

Starch granules consist of a mixture of amylose and amylopectin in proportions which vary from one starch to another. Amylose is essentially a linear polymer composed of  $\alpha$ -(1 $\rightarrow$ 4)-linked D-glucose while amylopectin is highly branched with  $\alpha$ -(1 $\rightarrow$ 4),  $\alpha$ -(1 $\rightarrow$ 6)-linkages (Briggs, 1978). In normal barley, about 22-30% of the starch is amylose and 70-78% is amylopectin (Morrison et al., 1986). However, the genetic variation in the amylose/amylopectin ratio is varied, including both high amylose and high amylopectin (waxy) genotypes. A high amylose level of 41-44% has been found in a mutant of Glacier (CI 9676) barley, which is designated Glacier Ac38 (Merritt, 1967). This character was shown to be under a single recessive gene (*amo1*) which is

located on chromosome 3 (von Wettstein-Knowles, 1992). Walker and Merritt (1969) reported this gene with a dose effect of allele. Endosperm which are triploid, were accordingly produced representing 0, 1, 2, and 3 doses of mutant allele by reciprocal hybridization between the mutant (Ac38) and the its parent, Glacier (CI9676). The high-amylose gene from high-amylose Glacier differs fundamentally from the *amylose extender* (*ae*), *dull* (*du*) and *sugary* (*su*) genes that increase the proportion of amylose to amylopectin in maize. In contrast, waxy starch, with amylose content as low as 0-8% (Morrison et al., 1986), is controlled by the recessive waxy endosperm gene *ww* in maize (Nakao, 1950) and barley (Goering and Eslick, 1976). The waxy barley was native to Asia (Banks et al., 1970), and the waxy gene in it was shown to be located on chromosome 1 (Hockett and Nilan 1985). This gene was introduced into two parent barley cultivars, Compana and Betzes (Goering and Eslick, 1976). Various other traits such as nude and short awn were also introduced, resulting in isogenic series. Åman and Newman (1986) first suggested that waxy barleys contain less starch and a greater quantity of sucrose, but did not measure maltose. Therefore, the waxy gene may affect not only the starch type but also other components of the waxy barleys, such as free sugar,  $\beta$ -glucan and dietary fiber (Newman and Newman, 1992).

Amylopectin in the starch granule constitutes the crystalline region, whereas amylose is mainly amorphous, filling the spaces between the clusters of amylopectin branches (Eliasson et al., 1987). The waxy varieties of starch give the same X-ray diffraction patterns as the normal starch, whereas high amylose varieties exhibit very low crystallinity. Further, amylose can be leached from the intact starch granule without

affecting the crystal state of the granule (Zobel, 1992). The consequence of the differences in structure between the two major constituents of starch is that their properties are quite different, as described by Marshall (1972).

### Some Properties of Starch

Starch granules are insoluble in cold water, but by heating above 50°C in excess water, the structure of the granule is altered by swelling, hydration and solubilization. French (1984) has theorized that the swelling of the amorphous phase contributes to the disruption of the crystalline regions by tearing molecules from the crystallites. This process called gelatinization involves melting of the crystallites that are present in the native granule. It is known that the gelatinization behavior of the starch granule is affected by its amylose content. According to several reports, the gelatinization temperature of maize and pea starches is generally increased (135°C to 150°C) at a higher amylose content (50% to 80%) as compared that of 75°C to 85°C in normal starches (Colonna and Mercier, 1985; Manners, 1985; 1988). It was demonstrated that a close correlation exists between the degree of starch gelatinization and the rate of enzymic hydrolysis both *in vitro* and *in vivo* (Holm et al., 1988). When gelatinized amylose starch is cooled, retrogradation may occur, causing a return from a solvated dispersed, amorphous state to an insoluble, aggregated or crystalline state. Retrogradation is partly reversible by heating the retrograded starch, which dissolves the crystalline nuclei. However, starch may retrograde so firmly that it becomes irreversible and totally resistant to  $\alpha$ -amylolysis and thus it can escape digestion both *in vitro* and *in vivo* (Englyst and Cummings, 1985; Björck et al., 1987; Ring et al., 1988). This enzyme

resistant starch (ERS) is generally defined as the component of native or processed starch that is able to survive exhaustive digestion with amylolytic enzymes, but which nevertheless can be measured as  $\alpha$ -glucan after solubilization with alkaline dimethyl sulfoxide (Berry, 1986). Processed foods such as bread, breakfast cereals and biscuits (Englyst and Cummings, 1985; Björck et al., 1986; Crawford, 1987;) or cooked amylo maize starch (Berry, 1986) contain appreciable quantities of ERS. The formation of this ERS is influenced by the amylose content, processing temperature and water content. The association between the amylose content and yield of ERS suggests that the linear components of starch and the heating process are involved, hence debranched amylopectin yielded 32-46% ERS by autoclaving and drying (Berry, 1986). Twenty cycles of autoclaving at 121°C-134°C with excess water and subsequent cooling increased the ERS formation up to 25% in high-amylose barley starch and 40% in amylo maize (Sievert and Pomeranz, 1989; Szczodrak and Pomeranz, 1991). Under these conditions, waxy maize starch yielded less than 1% resistant starch (Berry, 1986; Sievert and Pomeranz, 1989). This ERS is believed to consist mainly of retrograded amylose, as the amount formed increases with increasing amylose content (Russell et al., 1989; Berry, 1986; Ring et al., 1988; Siljeström et al., 1989; Szczodrak and Pomeranz, 1991). Since ERS is reported to have reduced bioavailability in the human gastrointestinal tract and to exert physiological effects similar as those of dietary fiber (Björck et al., 1987; Jenkins and Jenkins, 1985; Schneeman, 1989), it has been proposed that it be considered part of the total dietary fiber (Englyst and Cummings, 1987).

### Nutritional Implications of Starch

A wide variation in the digestibility of starch granules both *in vitro* and *in vivo*, depending on starch source, structure and food processing and storage conditions, have been reported (Dreher et al., 1984). The efficient digestion of starch has been shown to be critical for formula-fed infants 6 months of age or younger (Auricchio et al., 1967) as well as elderly people or those with medical disorders who have reduced digestive capacity (Sugimoto et al., 1980). In animal feedstuffs, maximum efficiency of feed utilization is needed to reduce production cost, and starch is the most important source of energy (Hale, 1973). On the other hand, a somewhat incomplete starch digestibility may be nutritionally advantageous in certain conditions. Malabsorbed starch reaching the colon could physiologically function like dietary fiber as mentioned above and have therapeutic applications such as blood glucose control in diabetics or as an aid in weight control (Jenkins et al., 1987).

Starch digestibility and absorption can be measured by different methods, each having its own advantages and limitations and each providing specific information. Both the rate and extent of starch hydrolysis *in vitro* are regarded as predictors of metabolic responses to complex carbohydrate *in vivo* (O'Dea and Holm, 1988). A commonly used method for the investigation of rates of starch hydrolysis in foods is simulated digestion *in vitro*. It has been well established that such differences correlate with postprandial blood glucose changes in humans under experimental conditions (Lund and Johnson, 1991; Brand et al., 1985; Bornet et al., 1989). The glucose tolerance test monitors the rise in plasma levels of glucose and insulin following oral ingestion of carbohydrate

meals. The rapidity, duration and the degree of rise of plasma glucose and insulin represent the end result of gastric emptying, digestion, absorption and also that of glucose utilization. Over the years, it has frequently been demonstrated that the ingestion of various types of starchy foods has various effects on the postprandial glucose and insulin responses in healthy and diabetic humans. These observations led to the development of the glycemic index which is defined as the ratio of the area under the incremental blood glucose response curve after 2 h for healthy subjects and 3 h for diabetics (Jenkins et al., 1981). The standard was originally pure glucose and later white bread was used (Wolever et al., 1986). The glycemic index obtained for the various starchy foods clearly shows that this index is affected by many factors including the source of the food, particle size and type of preparation dietary mixtures (Thorne et al. 1983). The chemical composition of the starch (amylose vs amylopectin ratio) appears to be an important influence (Björck et al., 1990).

Waxy starch from maize, barley and sorghum in the uncooked state has been shown to be more readily digested by  $\alpha$ -amylase *in vitro* than normal starch or high amylose starch (Sandstedt and Hites, 1968; Goering et al., 1973; Sullins and Rooney, 1974; Björck et al., 1990). However, this has not been observed *in vivo* when waxy barley starch was fed to rats (Calvert et al., 1976) or when whole grain waxy barleys were compared with normal barleys for ruminants (Moss et al, 1980), rats and swine (Calvert et al, 1977) and broiler chickens (Moss et al, 1983). The data from these studies varied with experiments and therefore left an open question about waxy starch digestibility.

High-amylose barley (HAG) and amylo maize starch were less susceptible to  $\alpha$ -amylase than normal starch (Pomeranz et al., 1972; Dreher et al., 1984). Calvert et al. (1976) reported that rats consumed less of a purified diet prepared with HAG starch than a similar diet containing Glacier starch and as a consequence gained at a slower rate. Amylo maize starch has lower blood glucose and insulin levels in humans (Behall et al., 1988; Behall and Scholfield, 1989; Amelsvoort and Weststrate, 1992) and high-amylose rice has similar effects (Goddard et al., 1984; Panlasigui, et al., 1992). Behall and Scholfield (1989) also reported a lowering of blood triglycerides and cholesterol in subjects consuming amylo maize starch and concluded that long-term intake of high-amylose starch may benefit hyperlipidemic individuals as well as those with adult-onset diabetes.

Available data on the nutritional properties of amylose starch are not consistent. Calvert et al. (1981) found that barley starch type did not significantly affect gain or feed consumption of swine. Rubin et al. (1974) demonstrated HAG to be nutritionally superior to five other barleys including Glacier as measured by growth of weanling rats. In a study with rice, a slightly higher amylose content (22 vs 15%) resulted in an increased rate of *in vitro*  $\alpha$ -amylolysis and an increase in postprandial glucose in healthy subjects (Srinivasa Rao, 1971).

Björck et al., (1990) reported that the amylose/amylopectin ratio in different barley genotypes (waxy, normal and high-amylose starch) produced no differences in *in vitro* digestibility when the barley flour was boiled even though results were significantly different when the raw starches were tested. Autoclaving the barley flour, however,

produced a concomitant decrease in *in vitro* digestibility with increased amylose content. This was explained by an increased amount of ERS (3%) and the formation of amylose-lipid complexes, which did not occur in autoclaved waxy barley or in any of the flours when boiled (Björck et al., 1990). Little is known about the long-term effects of amylose starch and the contribution of resistant starch to the total level of non starch-polysaccharides to human nutrition.

### Dietary Fiber

#### Composition of Dietary Fiber in Barley

Dietary fiber (DF) has been defined as non-starch polysaccharides (NSP) plus lignin (Theander et al., 1989) or plant materials resistant to hydrolysis by digestive enzymes of mammals (Hipsley, 1953). Plant cell wall materials are the major source of DF. Three main NSP were found in barley seed cell walls: arabinoxylans, cellulose and mixed linked (1→3), (1→4)- $\beta$ -glucans (Henry, 1988). Arabinoxylans are polysaccharides containing (1→4)- $\beta$ -xylan chains with arabinose residues attached through (1→2) or (1→3)-linkages. Cellulose is composed of linear (1→4)- $\beta$ -glucans. The  $\beta$ -glucans are also linear polymers and consist of  $\beta$ -cellotriosyl and  $\beta$ -cellotetraosyl residues separated by (1→3) linkages arranged in an independent or random way along the chain (Staute et al., 1983).

The composition of cell walls of barley seed can be quite different depending on the botanical tissue. Husk of barley grain consists of approximately equal amounts of arabinoxylans (50%) and cellulose residues (46%) (Salamonsson et al., 1980).

Arabinoxylans and  $\beta$ -glucans are concentrated in the aleurone and endosperm cell walls but the ratio between the two are different (Henry, 1988). Aleurone cell wall contains about 60% arabinoxylans and 20%  $\beta$ -glucans (Bacic and Stone, 1981) whereas endosperm cell walls contain 25% arabinoxylans and 70%  $\beta$ -glucans (Ballance and Manners, 1978).

The total  $\beta$ -glucan content of barley grain has been extensively studied and is shown to vary, depending on genotype or cultivars and the growing environmental conditions (Bourne and Pierce, 1970; Aastrup, 1979; Andersson et al., 1978; Newman and McGuire, 1985; Ullrich et al., 1986; Henry, 1986). Åman et al (1989) demonstrated an interaction of genotype x environment effect on  $\beta$ -glucan content of barley. Åman and Graham (1987) showed the average for Scandinavian barleys was 4.4% with a range of 3.0 - 5.6% of total  $\beta$ -glucan, of which 50-70% are soluble in water. Hockett et al. (1987) found a range in  $\beta$ -glucan of 4.9 - 6.2% with a mean of 5.5% in 12 North American cultivars over a 3-year period.

Powell et al. (1985) reported that the  $\beta$ -glucan content of barley is controlled by a simple additive genetic system. Waxy isotypes of Titan (CI 7055), Betzes (CI 6398) and Compana (CI 5438) barley contained 7.1, 7.3 and 4.7%  $\beta$ -glucan compared with those of 4.7, 5.7 and 3.8%  $\beta$ -glucan in non-waxy types (Ullrich et al., 1986). Azhul (a waxy hull-less six-rowed barley) was found to contain 10 - 11%  $\beta$ -glucan while the non-waxy parent barley contains 6-7% (Mori, 1990). The mechanism of the waxy gene effect on barley  $\beta$ -glucan content is currently unknown.

Total dietary fiber and soluble dietary fiber (SDF) in barley can be concentrated





















































































































































































