MONTANA STATE UNIVERSITY

The effect of the waxy endosperm, short awn, and hulless seed genes upon the biochemical and physiological seed characteristics important in barley (Hordeum vulgare L.) utilization

by Gregory Joseph Fox

A thesis submitted in partial fulfillment for the requirements for the degree of DOCTOR OF PHILOSOPHY in CROP AND SOIL SCIENCE
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Abstract:
The effect of the waxy endosperm, short awn, and hulless seed genes upon alpha-amylase activity in ungerminated seed, starch digestibility, starch and protein content, beta-glucan viscosity, and kernel weight was examined in the cultivars 'Compana', 'Titan', and 'Betzes'.

A gel-diffusion assay for alpha-amylase, based on a chromogenic substrate (amylose-azure blue) suspended in an agar medium was developed. Activity was measured as the diameter of digestion rings around sample wells into which a flour paste or enzyme extract was placed. The area of the digestion rings was proportional to the log10 of actual enzyme activity (r = .99**). Hundreds of samples could be assayed daily.

No unusual amount of alpha-amylase in nongerminated seed was found in any isotype within any cultivar. Some activity was found in all 50 barley cultivars and isotypes tested. No unique isozymes for alpha- and beta-amylase were found in any of the waxy, short-awn, hulless isotypes of Compana. However, the presence of the waxy gene was found to increase starch digestability. This was evident when upon malting, waxy isotypes produced higher levels of reducing substances (maltose) at a more rapid rate than normal isotypes.

Kernel weights were reduced for all mutant isotypes while beta-glucan viscosity was increased. In nonwaxy isotypes, these increases were proportional to the decreases observed for kernel weight. Waxy isotypes had viscosity levels significantly higher than that predicted by kernel weight reductions indicating they were accumulating beta-glucans beyond the simple concentration effect of reduced kernel weight. Waxy isotypes had lower extract (starch content) and higher protein.

All isotypes responded across environments and genotypes in a relatively uniform manner for extract and protein. Regression analysis demonstrated that the background genotype and environment can modify the kernel weight and beta-glucan response of a particular isotype. When tested in the Brabender Amylograph, waxy, waxy hulless, waxy short-awn, and waxy short-awn hulless isotypes within Titan, Betzes, and Compana pasted in the same temperature range (65-75 C). Pasting peak viscosity was variable, but related to alpha-amylase activity and beta-glucan viscosity of the barley flour samples (r = .96**).

THE EFFECT OF THE WAXY ENDOSPERM, SHORT AWN, AND HULLESS SEED GENES UPON BIOCHEMICAL AND PHYSIOLOGICAL SEED CHARACTERISTICS IMPORTANT IN BARLEY (*HORDEUM VULGARE* L.) UTILIZATION

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ABSTRACT

The effect of the waxy endosperm, short awn, and hulless seed genes upon alpha-amylase activity in ungerminated seed, starch digestibility, starch and protein content, beta-glucan viscosity, and kernel weight was examined in the cultivars 'Compana', 'Titan', and 'Betzes'.

A gel-diffusion assay for alpha-amylase, based on a chromogenic substrate (amylose-azure blue) suspended in an agar medium was developed. Activity was measured as the diameter of digestion rings around sample wells into which a flour paste or enzyme extract was placed. The area of the digestion rings was proportional to the $\log_{10}$ of actual enzyme activity ($r = .99^{**}$). Hundreds of samples could be assayed daily.

No unusual amount of alpha-amylase in nongerminated seed was found in any isotype within any cultivar. Some activity was found in all 50 barley cultivars and isotypes tested. No unique isozymes for alpha- and beta-amylase were found in any of the waxy, short-awn, hulless isotypes of Compana. However, the presence of the waxy gene was found to increase starch digestability. This was evident when upon malting, waxy isotypes produced higher levels of reducing substances (maltose) at a more rapid rate than normal isotypes.

Kernel weights were reduced for all mutant isotypes while beta-glucan viscosity was increased. In nonwaxy isotypes, these increases were proportional to the decreases observed for kernel weight. Waxy isotypes had viscosity levels significantly higher than that predicted by kernel weight reductions indicating they were accumulating beta-glucans beyond the simple concentration effect of reduced kernel weight. Waxy isotypes had lower extract (starch content) and higher protein.

All isotypes responded across environments and genotypes in a relatively uniform manner for extract and protein. Regression analysis demonstrated that the background genotype and environment can modify the kernel weight and beta-glucan response of a particular isotype.

When tested in the Brabender Amylograph, waxy, waxy hulless, waxy short-awn, and waxy short-awn hulless isotypes within Titan, Betzes, and Compana pasted in the same temperature range (65-75 C). Pasting peak viscosity was variable, but related to alpha-amylase activity and beta-glucan viscosity of the barley flour samples ($r = .96^{**}$).

CHAPTER 1

INTRODUCTION

Barley (Hordeum vulgare L.) is an important cereal grain in Montana and the Western United States. It is generally classified as a feed grain, and malting is the only premium market that exists. In order to improve the producer's cash return on barley, researchers at Montana State University have emphasized the development of alternate uses. One such use is the production of high maltose syrup from waxy, short-awn, hulless barley.

The purpose of these investigations was to determine how the waxy, short-awn, and hulless genes, separately and in combination, affect seed quality characteristics that influence high maltose syrup production. Characters such as alpha-amylase activity, starch digestibility, and beta-glucan viscosity, affect the ease of syrup production. The relative proportions of seed components such as protein, starch, and beta-glucan, influence the total amount of syrup produced. The effect of the three genes on these characters was examined within three genotypic backgrounds across a range of environments.

Although these investigations have focused on high maltose syrup production, they may also provide useful information for other industrial and domestic uses of barley, such as, malting and brewing,

milling and baking, ethanol production, and human and animal nutrition.

CHAPTER 2

LITERATURE REVIEW

The Use of Washonupana Barley in High Maltose Syrup Production

The barley cultivar 'Washonupana' is a triple isogenic of the cultivar 'Compana'. It was developed by the selection of waxy endosperm, short-awn, hulless lines from the cross, waxy 'Oderbrucker'/7*Compana/2/short awn hulless/7*Compana (Goering and Eslick, 1976). Thus, the name Washonupana is an acronym for waxy (wa), short awn (sho), nude (nu), Compana (pana).

The starch extracted from Washonupana barley was reported to be unique because it retained a sufficient amount of alpha-amylase to liquefy itself in a Brabender Amylograph at 70 C (Goering and Eslick, 1976). This liquefaction occured without the addition of any amylolytic enzymes. This is unique because sound barley is reported to have little or no alpha-amylase at harvest (Kneen, 1944). The self-liquefying property of Washonupana barley prompted its use as the first experimental high maltose syrup barley (Goering et al., 1981).

The high maltose syrup process (Goering et al., 1981) initially separates Washonupana barley grain into the various seed components. The grain is first milled to produce flour which is then suspended in warm water (40-45 C). The crude fiber and aleurone protein fraction

is screened off. The crude starch fraction which consists of starch, endosperm storage protein, and the warm water soluble gums (beta-glucans and arabinoxylans derived from cell wall material) is re-suspended. This mixture is pasted (10 minutes at 75 C) to disrupt all the starch granules then cooled to 40 C in order to add green malt which saccharifies the starch. After four hours the starch is solubilized and the suspended protein and beta-glucan material is removed from solution by centrifugation of the solids. The supernatant is then heated to 60 C and conversion to sugar is complete within 24-36 hours.

The syrup making process is similar to the production of sweet wort in the brewing industry. The main difference is that it produces high maltose syrup, 70% protein, bran fiber plus protein, and possibly beta-glucan as separate end-product fractions (Goering et al., 1981). The brewer, on the other hand, produces a heterogeneous sweet wort that contains sugars, dextrins, proteins, amino acids, and gums (Palmer and Bathgate, 1976). The solid residue (spent grain) from the brewing process is an equally heterogenous mixture of starch, gums, protein, crude fiber, and sugars which is usually dried and marketed as animal feed (Pomeranz, 1973).

One problem encountered in the Goering syrup process is the occurence of high levels of beta-glucans or gums in Washonupana barley

grain (Goering and Chapman, personal communication). In the initial isolation of the crude starch fraction, the gum becomes dissolved or suspended in the warm water mixture, increasing the viscosity and impeding starch separation. Thus, in order to effect efficient starch separation, it has been necessary to add beta-glucanase to reduce the viscosity of the warm water grist (Goering et al., 1981). These gums have potential as industrial gelling agents (Wood, personal communication), however, it is difficult to separate them from the protein. Thus, it is not clear whether they should be separated with the protein from the crude starch or simply hydrolyzed and allowed to be a component of the finished syrup (Chapman, personal communication).

The Pasting Characteristics of Starch as Measured by the Brabender Amylograph

Starch granules are insoluble in cold water, but as starch and water is heated, the granules absorb water and swell. This swelling is reversible until the gelatinization temperature is reached. Structural integrity of the granule is lost at this temperature and material is leached from the granule. Gelatinization is followed by loss of birefringence, increased susceptibility of the granule to enzymatic degradation, and increased viscosity of the starch solution. After reaching peak viscosity, the granules completely break down and viscosity is greatly reduced (Greenwood, 1976).

The swelling and subsequent disruption of granules is referred to as "pasting" and is measured in a Brabender Amylograph (Smith, 1964). This instrument measures the viscosity (as Brabender Units, B.U.) of an aqueous starch suspension as it is mechanically stirred and heated according to a preset time/temperature schedule. The temperatures which bring about initial gelatinization, pasting peak, and liquefaction are determined with this instrument (Smith, 1964 and Goering et al., 1973a).

In addition to purified starch, crude flour (Goering et al., 1981) and malt flour (Yoshida and Yamada, 1970) have been studied with the Brabender Amylograph. Adding alpha-amylase to crude barley flour and purified barley starch greatly altered the initial pasting temperature, pasting peak temperature, and final liquefaction temperature and viscosity (Yamada and Yoshida, 1973). The addition of beta-1, 4 glucanase to crude barley flour greatly reduced the overall viscosity of the mash, while the addition of protease had no appreciable effect. Goering and Eslick (1976) observed that the presence of alpha-amylase with Washonupana starch reduced peak viscosity and resulted in complete liquefaction of barley starch (B.U. = 0). The observed initial pasting temperature was not affected.

Genetic Components of Washonupana Barley

The three genes that were introduced into Compana barley to produce Washonupana barley are all recessive and located on chromosome 1.

The waxy gene (wxwx) is located on the short arm approximately 50 recombination units from the centromere (Nilan, 1964). The short-awn (lk2lk2) and the hulless gene (nn) are linked characters located on the long arm. The hulless gene is about 7 recombination units from the centromere while the short-awn gene is about 15 recombination units from the centromere (Eslick et al., 1972).

Waxy Endosperm

The waxy endosperm gene was introduced into syrup barley cultivars because of the observation that waxy starch is more readily modified by enzymes and chemicals than normal starch (Cowie and Greenwood, 1957; Buttrose, 1960; Leach and Schoch, 1961; Sandstedt et al., 1968; Goering et al., 1973a; Sullins and Rooney, 1975; Tovar et al., 1977; and MacGregor and Ballance, 1980). Also, it has been found that waxy barley starch will paste at a temperature 20 C below normal starch in a Brabender Amylograph (Goering et al., 1973a; Goering and Eslick, 1976; and Goering et al., 1981). This represents savings in energy since starch must be pasted before it can be converted to sugar.

Normal starch found in barley and other small grains is a mixture of two polymers: a helical straight-chained polymer called amylose in which glucose units are joined by alpha-1, 4 linkages; and a branched polymer called amlopectin in which glucose units are joined by alpha-1, 4 and alpha-1, 6 linkages. The branches of amylopectin are formed by

alpha-1, 6 linkages and are 20-24 glucose molecules long (Harris, 1962a; Banks and Greenwood, 1975; and Greenwood, 1976).

Waxy and normal grain differ in that the waxy type produces little (0-3%) of the straight chained amylose (Goering et al., 1973a and Briggs, 1978). Badenhuizen (1955, 1963) reported that in waxy maize a very small amylose portion was localized in the center of the granule. Normal barley starch consists of 15-25% amylose and 75-85% amylopectin and is not markedly affected by environment (Goering et al., 1957). This ratio is typical for most cereals (Harris, 1962a; Creech, 1968; and Banks and Greenwood, 1975).

Waxy starch can be distinguished from normal starch by iodine staining. Iodine reacts with the long chained amylose to produce a deep blue color while the reaction with the short chained (20-24 glucose residues) amylopectin produces a red color (Harris, 1962a; Creech, 1968; and Banks and Greenwood, 1975).

The occurrence of waxy endosperm starch in corn and rice is closely associated with an observed deficiency or complete lack of granule-bound UDP-G and ADP-G starch synthetase. Waxy corn and rice do possess soluble starch synthetase (Nelson and Rines, 1962; Nelson and Tsai, 1964; Akazawa and Murata, 1965; Murata et al., 1965; Frydman, et al., 1966; Murata and Akazawa, 1966; and Creech, 1968).

Starch granule synthesis occurs in amyloplasts within the barley

or cereal endosperm. The starch granules are laid down by apposition, layer by layer (Badenhuizen and Dutton, 1956 and May and Buttrose, 1959), forming a semi-crystalline structure with concentric amorphous and crystalline portions (Buttrose, 1960; Banks and Greenwood, 1975; and Greenwood, 1976).

Starch granule ultrastructure is solely dependent upon amylopectin. Amylose can be completely leached from starch granules allowed to remain in an aqueous solution at or near the gelatinization temperature (barley = 65 C). Under these conditions, the amylose will dissolve into the water while the crystalline structure of the granule remains intact. Waxy granules, even though completely lacking in amylose, form normal semi-crystalline granules (Montgomery and Senti, 1958; Banks and Greenwood, 1975; and Greenwood, 1976). Buttrose (1960) has noted that amylose has the effect of hardening the amylopectin granule against acid hydrolysis. It appears to intermesh with the granule-forming amylopectin to produce a more tightly packed starch granule.

The waxy gene produces no gross noticeable difference in crop appearance except that it imparts to the grain an opaque hue in contrast to the more vitreous appearance of normal grain (Ericksson, 1969). This characteristic is not readily apparent in hulled barley but is easily observed in hulless types.

Some physiological differences between normal and waxy types have been observed. Creech (1968) reported that waxy maize isotypes had reduced dry matter accumulation at all stages of development. Also, reduced endosperm starch accumulation was observed but not to a significant level. Eslick (1979) observed slight but insignificant yield reductions for the waxy barley types when compared to normals. When waxy corn isotypes were first introduced there were significant yield reductions. Improving the genetic background of the waxy lines resulted in yield levels comparable to normal maize cultivars (Crookston, 1979).

Bruckner and Eslick (1979) observed that the waxy isotype of Compana had less field dormancy and higher levels of alpha-amylase at all stages of seed development. MacGregor and Ballance (1980) reported that waxy starch granules were more susceptible to hydrolysis than normal starch granules. They suggested that the unique attached enzyme phenomenon of Washonupana barley was due primarily to the waxy nature of the starch.

Waxy grains have been evaluated for feed value since waxy starch was shown to be more digestible in vitro than normal starch (Sullins and Rooney, 1975). In studies with sorghum, advantages in feed efficiency of waxy grain compared to normal sorghum were observed for sheep (Nishimuta et al., 1969) and steers (Sherrod et al., 1969).

However, Cohen and Tanksley (1973) found no difference in the performance of swine fed waxy vs. nonwaxy grain sorghum. Superior performance of swine (Hanson, 1946) and ruminants (Braman et al., 1973) raised on waxy corn has been reported. But, other investigations have shown no appreciable advantage for ruminants (McCormick and Farlin, 1974 and Robinson et al., 1974) fed waxy vs. nonwaxy corn. Swine (Calvert et al., 1977), dairy cattle, beef cattle, and sheep (Moss et al., 1980), performed equally when fed waxy and nonwaxy barley rations.

Short Awn

Awns are active photosynthetic organs in small grains (Grundbacher, 1963). As awn length is reduced, photosynthetic rate declines (Johnson et al., 1975). Shannon and Reid (1976) reported yield reductions in comparisons of awnless vs. full-awned isogenic lines of winter barley. In studies with full-awned, half-awned, quarter-awned, and awnless isogenic barley lines, Schaller et al. (1972) observed kernel weight reductions proportional to the reduction in awn length. Significant yield reductions were also observed but only within high yielding environments. In comparison of short-awn (lk2lk2) vs. long-awn (Lk2Lk2) isotypes of hulled and hulless barley, McGuire and Hockett (1981) found that kernel weights were significantly reduced in the short-awn isotypes. However, awn length had no significant effect on

yield. Eslick (1979) found no yield differences in isogenic comparisons of long-awn vs. short-awn isotypes grown in Montana environments. He suggested that while kernel weight is reduced in short-awn isotypes, yield remains unchanged because these isotypes compensate by producing more seed.

Barley endosperm contains a bimodal distribution of starch granules (May and Buttrose, 1959 and Goering et al., 1973b). MacGregor (1979) divided the two groups of granules using a 5.3 um diameter as the dividing point, with large granules being greater than 5.3 um and small granules smaller than 5.3 um. The small granules are difficult to separate in the Goering syrup process because they can be washed away in the mill water or remain trapped in the gum-protein complex (Goering et al., 1981). In general, 90% of the starch granules are the small type but they represent only 10% of the starch present in the barley (May and Buttrose, 1959 and Goering et al., 1973b). The short-awn trait was included in the development of the syrup barley Washonupana because the short-awned isotype of Compana contained a high proportion of easy to process large starch granules (Goering et al., 1973b).

Hulless Seed

In crops such as barley and rice, the flowering glumes (the lemma and palea of the barley flower) are cemented to the caryopsis

to produce a hulled grain. In hulless isotypes, the hulls are not cemented to the seed (Briggs, 1978). Kernel weight and yield reductions of hulless isotypes are proportional to at least the weight of the hulls (8-16% of the kernel weight) (Briggs, 1978 and Eslick, 1979). Since the hulls are left in the field, the proportion of other seed components changes accordingly. The fat, protein, and starch content of the seed increase due to the reduction in the crude fiber represented by the hulls (McGuire and Hockett, 1981).

The hulless-seed trait has appeal in the barley syrup process because of reduced amounts of harsh lignified fiber, most of which is contained in the hulls of barley (Briggs, 1978). Goering et al. (1981) stated that the hulls only serve as an unwanted nuisance in the production of syrup from barley.

Alpha-Amylase in Barley

Alpha-amylase is the main saccharifying enzyme of cereals. It hydrolyzes the alpha-1, 4 linkage of both amylose and amylopectin in a random manner. The importance of alpha-amylase lies in the fact that it is the only enzyme capable of digesting starch granules (Harris, 1962b; Dunn, 1974; and Maeda et al., 1978).

Under natural conditions, alpha-amylase does not work alone. It functions in breaking the integrity of the starch granule and reduces the starch into segments of 6-8 glucose molecules. Two other enzymes

are necessary to complete the conversion of starch into maltose and other sugars. Beta-amylase, which attacks a nonreducing end and breaks off two glucose molecules (maltose) at a time, converts the fragments produced by alpha-amylase to maltose. The third enzyme, pullanase, is necessary to hydrolyze the alpha-1, 6 linkages of amylopectin before complete starch hydrolysis can occur (Harris, 1962b; Dunn, 1974; and Palmer and Bathgate, 1976).

Alpha-amylase is a unique enzyme in that it has a remarkable temperature range. Other hydrolytic enzymes of barley (beta-amylase, beta-glucanase, protease, and pullanase) have their optimum temperature range from 40-50 C. The optimum range for alpha-amylase is 50-55 C, and the enzyme will function quite well at 70 C (although at reduced efficiency), a temperature at which all the other hydrolytic enzymes are completely denatured (Olson et al., 1943; Harris, 1962a; Harris, 1962b; and Palmer and Bathgate, 1976).

Alpha-amylase is a complex mixture of isozymes. The number of isozymes detected often depends on the barley cultivar examined, the environment in which it is grown, and the method used to separate the isozymes (MacGregor, 1978 and MacGregor and Dussant, 1979). However, there are two basic types of alpha-amylase that have a physiological as well as chemical distinction. Green alpha-amylase, or alpha-amylase I, is primarily found in developing seed. It develops in the pericarp

prior to anthesis and reaches a peak at 2-10 days after anthesis. The level of alpha-amylase activity in the developing seed never exceeds more than 1-5% of the activity of malted grain. As the seed reaches physiological maturity the activity is rapidly reduced until at harvest only small amounts of activity remain. The function of alpha-amylase I is unknown, but it has been suggested that starch granules found in the green pericarp tissue are hydrolyzed to provide energy for growing kernels or produce short chain primers to be used in endosperm starch synthesis (MacGregor et al., 1971a and MacGregor et al, 1972).

The other major component of alpha-amylase is germinative alpha-amylase or alpha-amylase II and III. These isozymes are produced de novo by germinating seed (Varner and Chandra, 1964; MacGregor et al., 1971b; and MacGregor, 1976). In order for germination to occur the moisture level of the seed must reach 35%. At this point the embryo begins to respire and secretes gibberellic acid which moves to the aleurone layer where it triggers the production of alpha-amylase. This is the sequence of events in the production of all the major hydrolytic enzymes except beta-amylase which is preformed in the endosperm as part of the protein matrix (Harris, 1962a; Harris, 1962b; and Palmer and Bathgate, 1976). The main purpose of malting in the brewing industry is the production of these hydrolytic enzymes, es-

pecially alpha-amylase, through controlled steeping and germination (Pomeranz, 1973).

Numerous methods have been used to measure alpha-amylase in cereals, but they are all based upon the same principle, action of alpha-amylase enzyme upon a substrate. The reduction in viscosity of a starch or flour solution can be measured using the Falling Number Method (Hagberg, 1961 and Perten, 1964). Particle size reduction of a dextrin substrate can be measured optically using the Nephalometric technique (O'Connell et al., 1980). Decreased iodine color of an iodinated beta-limit dextrin can be measured colorimetrically with the Iodine Dextrin Color Loss Method (Sandstedt et al., 1939). The increase in reducing power that occurs with hydrolysis of alpha-1, 4 linkages of starch can be measured by titration or quantitative dye attachment to the reducing ends of dextrin fragments with the Reducing Sugars Method (Hodge and Hofreiter, 1962).

In the last 10 years, cross-linked dye-labelled starch substrates that are quite specific for alpha-amylase have become available (cibacron blue amylose, amylose-azure blue). These substrates are dissolved in solution. With the addition of amylase, the starch is hydrolyzed and the dye released. The amount of dye released is quantitatively related to the alpha-amylase activity and can be measured with a spectrophotometer (Klein et al., 1969; Barnes and Blakeney,

1971; and Mathewson and Pomeranz, 1977). These chromogenic substrates can also be employed in gel-diffusion assays in which activity diameters are related to enzyme activity (Hejgaard and Gibbons, 1979).

Beta-Glucan in Barley

The starchy endosperm cell walls of barley are composed largely of a polymer called beta-glucan (75%) (Fincher, 1975; Forrest and Wainwright, 1977a; and Forrest and Wainwright, 1977b). Beta-glucans are straight chained beta-linked glucose units linked at the 1,4 position (as in cellulose) and at the 1,3 position. The average proportion of 1,3 to 1,4 linkages is 3:7 (Parish et al., 1960 and Bourne and Pierce, 1972).

The other polysaccharide components of the cell wall are pentosans, such as, arabans, xylands, and arabinoxylans. These polysaccharides are similar to the beta-glucans but the components are 5 carbon sugars and there is some evidence of branching within the polymer. Pentosans are the principle cell wall constituent in the aleurone layer (McNeil et al., 1975 and Palmer and Bathgate, 1976).

There is also a protein fraction within the cell wall material. Since calcium pectate is lacking in the barley endosperm it has been suggested that cell wall protein serves as the cementing substance which binds endosperm cells together (MacLeod and McCorquodale, 1958 and Palmer and Bathgate, 1976).

In the past, the beta-glucans and pentosans were classified by their solubility properties. Components which dissolved in warm water are called gums; and those that were extracted by 4% sodium hydroxide solution were called hemicellulose (Harris, 1962a and Palmer and Bathgate, 1976). Current thinking is that gums and hemicellulose are all derived from the same basic source, cell wall polysaccharides. The difficulty of extraction is simply a measure of polymer chain length, difference in the proportion of beta-1,3 and beta-1,4 linkages, and/or location within the cell wall (Fleming et al., 1974; Bathgate and Dalgliesh, 1975; and Forrest and Wainwright, 1977a).

Beta-glucans have presented problems in the brewing industry by causing increased viscosity to the sweet wort which causes filtration problems. Excessive beta-glucan can cause haze formation and palatability problems in beer. Conversely, a certain amount of this substance is desirable in the beer since it provides body and foam stability (Forrest and Wainwright, 1977a).

The viscosity of a warm water beta-glucan extract prepared from barley flour is not strictly related to the beta-glucan viscosity of the sweet wort which is derived from malt flour. The malting process releases additional beta-glucans as cell walls are hydrolyzed and it is this material that creates a viscous sweet wort (Harris, 1962a; Scott, 1972; Bourne and Pierce, 1972; and Palmer and Bathgate, 1976).

The amount of beta-glucan that is found in barley grain has a very strong genetic and environmental component. While there is a great deal of variability across environments the relative performance of genotypes is constant (Bourne and Pierce, 1970; Bourne and Pierce, 1972; and Coles, 1979). The high lysine mutant 'Riso 1508' has extremely low levels of beta-glucan and is almost insensitive to normal environmental variation (Coles, 1979). The high protein oat (Avena sativa L.) cultivar, 'Hinoat', has abnormally high levels of beta-glucan (Wood et al., 1977).

Elevated beta-glucan content is associated with xerophytic conditions (Whitmore and Sparrow, 1957; Preece et al., 1958; and Bendelow, 1975). Coles (1979) reported that under drought conditions beta-glucan synthesis is favored over starch. When the moisture level of the developing seed is above 45%, the synthesis of starch from glucose is the dominant glucan consuming process. However, when the moisture level falls below 45%, starch synthesis is halted while beta-glucan synthesis increases sharply. In rain (sprinkler) vs. root irrigated plants, beta-glucan content of the grain of the latter is higher throughout development (Coles, 1979). No evidence of beta-glucan degradation in the seed of rain irrigated plants suggests that less beta-glucan is synthesized (Aastrup, 1979). This difference is

attributed to seed dessication which inhibits starch synthesis and favors synthesis of beta-glucans.

Aside from providing cell wall material to the endosperm, beta-glucan molecules can also serve as an alternate storage polysaccharide and as a water-conserving mechanism (Aastrup, 1979). Beta-glucan is hydroscopic (Preece and MacKenzie, 1952 and Greenberg, 1974) and up to 50% of the beta-glucan present in barley can be leached out before cell wall disintegration becomes apparent (Forrest and Wainwright, 1977b).

Gohl (1977) and Aastrup (1979) found that barley seed harvested at yellow ripeness had a more viscous extract than barley seed harvested at complete ripeness. However, total beta-glucan content did not change. Soluble beta-glucan decreased while the insoluble beta-glucan content increased (insignificantly). This suggests that as seed ripens the soluble beta-glucans which are the source of extract viscosity become more tightly bound within the cell wall and are rendered insoluble

The amount of beta-glucan reported in barley seed varies from 1.5 - 8.0% (Prentice et al., 1980). This variation is partly due to the assay used to measure this nonstarchy polysaccharide. Extraction procedures vary from assay to assay. If only the hot water soluble gums are extracted, the beta-glucan estimates tend to be low. Esti-

mates increase if both the water soluble fraction and the insoluble fraction are extracted using strong acid or basic solutions (Forrest and Wainwright, 1977a and Wainwright, 1978). Temperature of extraction also influences the estimation of beta-glucan content. Extraction in basic solution at 80 C solubilizes three times as much beta-glucan as extractions performed at 45 C (Prentice et al., 1980).

Basically there are two methods used to measure beta-glucan content of barley grain: (1) extraction of the beta-glucan with subsequent hydrolysis of the polysaccharide to glucose; and (2) extraction of the beta-glucan in a highly acidic or basic solution and measurement of viscosity of the extract (Wainwright and Buckee, 1977). The first method is the most accurate. Glucose content is measured optically (600 nm) by addition of a glucose oxidase reagent, and beta-glucan content is determined on a percentage basis. The actual hydrolysis of beta-glucan to glucose is achieved through glucanase enzymes (Prentice et al., 1980), or a combination of acid hydrolysis and enzyme hydrolysis (acid hydrolysis-amyloglucosidase procedure) (Fleming et al., 1974 and Wood et al., 1977).

The second method, viscosity, although less accurate, is much faster. The amount of beta-glucan is not measured directly, but as the amount of viscosity produced by a beta-glucan extract (Greenberg and Whitmore, 1974 and Morgan and Gothard, 1977). Vis-

cosity is related to actual beta-glucan content in a logarithmic fashion. Viscosity increases logarithmically as beta-glucan content increases arithmetically (Greenberg, 1974). Unfortunately, both quantity and quality of beta-glucan can affect viscosity. The larger molecules of beta-glucan have greater influence on viscosity than the small types (Bourne and Pierce, 1970). Also, the pentosan fraction influences viscosity (Bourne and Pierce, 1970; Bourne and Pierce, 1972; and Bathgate and Dalgliesh, 1975). Researchers using this method in the past, have found it to correlate well with actual beta-glucan content ($r = .89$) (Greenberg, 1974). However, this method is more appropriate when the experimenter is interested in viscosity rather than actual beta-glucan content of barley grain.

Recently an infrared reflectance procedure has been introduced for beta-glucan analysis using a Technicon Infraalyzer 400. This highly automated method determines the beta-glucan content by scanning ground flour samples. This method correlates well with both viscosity ($r = .81$) and actual percentage beta-glucan ($r = .87$). While this technique is recommended only for the screening of barley material, its potential as an analytical tool should not be overlooked (Allison et al., 1978).

Starch and Protein Accumulation in Developing Barley

Dry matter accumulation in developing barley seed closely follows the accumulation of the two major components of the endosperm: protein and starch (MacGregor et al., 1971a). The endosperm is primarily a storage organ which, with the exception of the aleurone layer, is dead tissue at maturity. The endosperm consists of starch granules (large and small) and protein bodies embedded in a protein matrix surrounded by a beta-glucan/arabinoxylan cell wall (Palmer and Bathgate, 1976 and Briggs, 1978). The cell walls of the endosperm function primarily as a structural component, however, as mentioned previously, they may function as carbohydrate storage components under certain environmental conditions (Coles, 1979). Starch, the chief source of stored reserves, comprises 50-65% of the seed weight. Overall yield is primarily determined by this seed component (Palmer and Bathgate, 1976 and Briggs, 1978).

Protein has several functions within the endosperm. The metabolically active proteins of the aleurone layer are primarily enzymes and enzyme precursors consisting of water soluble albumins and salt soluble globulins (Simmonds and Orth, 1973). Within the starchy endosperm there exists two types of protein. The 70% ethanol-soluble hordeins, which are purely storage proteins, contain a preponderance of glutamic acid and proline. Hordeins are the main

constituents of the protein bodies (Cameron-Mills et al., 1980). The glutelins are also thought of as storage structures in the endosperm, but also include a high percentage of membrane proteins (Simmonds, 1972). The glutelins are the primary constituents of the protein matrix which engulfs protein bodies and starch granules alike (Ingversen, 1975).

High protein levels in barley are derived from two sources, genotype and environment. Some cultivars and isotypes, such as 'Hiproly Normal' (Munck et al., 1971), have unusually high levels of protein regardless of environment (Scholz, 1976).

Certain environmental conditions favor protein accumulation in barley. Low yielding environments (drought or inadequate grain filling period) characterized by poor starch accumulation result in poorly filled, steely seed that are high in protein (Hunter, 1962 and Briggs, 1978). The increased protein content is due to reduced starch accumulation. High protein can also be caused by moderate to high-yielding environments in which excessive soluble nitrogen is available at grain filling. The elevated protein content is due to increased synthesis of protein (hordeins and glutelins) (Bishop, 1928; McBeath and Toogood, 1960; and Harris, 1962a). Elevated levels of these proteins are a detriment to malting and brewing and the industry encourages growers of malting cultivars to avoid these conditions

(Brewing and Malting Barley Research Institute, 1977).

Genetic and environmental factors also determine the starch content of barley. The cultivar, 'Karl', is noted for low protein production and high starch accumulation (Burger et al., 1979 and Wesenberg et al., 1981). Environmentally favorable growing conditions such as sufficient water and early planting produces barley grain with moderate to high levels of starch for the same reason that the reverse produces high protein barley with low yield. While high nitrogen is desirable at tillering, at seed filling starch accumulation is enhanced by low soluble nitrogen and adequate water (Foote and Batchelder, 1953 and Zubriski et al., 1970). This is the recommended environment for producing premium malt barley (Harvey, 1977).

The Effect of Environment on Genotypes and Isotypes

The effect that environments have upon isotypes or genotypes may be studied in a number of ways. The genotype x environment (G x E) and isotype x environment (I x E) interactions in an analysis of variance table is one possible method. However, it is quite difficult to visualize the nature of an interaction by noting the interactional component of an analysis of variance table when more than two environments, genotypes, or isotypes are involved.

Regression analysis gives the researcher a visual representation of the interactions within an experiment. Mooers (1921) was the first to apply regression analysis to study the environmental response of genotypes of corn. This approach has been advanced in recent years by Finlay and Wilkinson (1963) with barley, Eberhart and Russell (1966) with corn, and Bilbro and Ray (1976) with cotton. The basic objective of these researchers was to identify superior yielding genotypes for use over a variety of environments. The main factors of interest were environmental stability and the adaptability of the analyzed genotypes.

The first use of this type of analysis for I x E interactions was by Eslick and Hockett (1977) for two erectoides mutants of barley. Eslick and Ullrich (1977) expanded the use of this analysis to non-agronomic traits in a study of the lysine response of shrunken endosperm, high lysine mutants across diverse environments.

Ramage (1979) has suggested that for a single isotype the genotype x isotype interaction represents a legitimate environment x isotype interaction. He maintains that the specific genotype a gene occupies is a genuine environment and should be examined along with the physical environment for significant interactions.

Isotypes and Name Coding

The waxy gene, wxwx, was incorporated into three cultivars, Betzes, Compana, and Titan, by crossing to 'Waxy Oderbrucker', C.I. 7563, followed by six backcrosses to the recurrent parent (Eslick, personal communication). The short-awn, lk2lk2, and hulless seed, nn, genes were donated by the cultivar, 'Sermo', C.I. 7776. The short-awn, the hulless, and the short-awn, hulless isotypes were selected while backcrossing to Betzes, Compana (Hockett, 1981), and Titan (Eslick, personal communication), six times.

The waxy, hulless isotype of the three cultivars was selected in the F_2 after crossing the waxy isotype of a cultivar with the hulless isotype of the same cultivar. The waxy, short-awn isotype of the three cultivars was selected in the F_2 after crossing the waxy isotype of a cultivar with the short-awn isotype of the same cultivar. The waxy, short-awn, hulless isotype of the three cultivars was selected in the F_2 after crossing the waxy isotype of a cultivar with the short-awn, hulless isotype of the same cultivar (Eslick, personal communication).

A short-hand notation was devised to name all of the described isotypes (Eslick, 1979). The nature of the isotype is described by a prefix (Table 2-1) which is attached to some portion of the cultivar name (Table 2-2). A complete list of the waxy, short-awn hulless isogenic series of Betzes, Compana, and Titan is in Table 2-3.

Table 2-1. Gene description, symbol, and name code prefix used in naming isogenics.

Gene Description	Gene Symbol	Name Code Suffix
waxy endosperm	wxwx	wa-
short awn	lk2lk2	sho-
hulless (nude) seed	nn	nu-

Table 2-2. Cultivars and their name code suffix used in naming derived isogenics.

Cultivar	C.I. Number	Name Code Suffix
Betzes	6398	-bet
Compana	5438	-pana
Titan	7055	-tan

Table 2-3. The waxy, short-awn, hulless isogenic series of Betzes, Compana, and Titan.

Cultivar	C.I. Number	Genetic Description
Betzes	6398	WxWx Lk2Lk2 NN
Nubet	16559	WxWx Lk2Lk2 nn
Shobet	16558	WxWx lk2lk2 NN
Wabet	-	wxwx Lk2Lk2 NN
Shonubet	16560	WxWx lk2lk2 nn
Wanubet	-	wxwx Lk2Lk2 nn
Washobet	-	wxwx lk2lk2 NN
Washonubet	-	wxwx lk2lk2 nn
Compana	5438	WxWx Lk2Lk2 NN
Nupana	16185	WxWx Lk2Lk2 nn
Shopana	16188	WxWx lk2lk2 NN
Wapana	-	wxwx Lk2Lk2 NN
Shonupana	16186	WxWx lk2lk2 nn
Wanupana	-	wxwx Lk2Lk2 nn
Washopana	-	wxwx lk2lk2 NN
Washonupana	-	wxwx lk2lk2 nn
Titan	7055	WxWx Lk2Lk2 NN
Nutan	16494	WxWx Lk2Lk2 nn
Shotan	16496	WxWx lk2lk2 NN
Watan	-	wxwx Lk2Lk2 NN
Shonutan	16495	WxWx lk2lk2 nn
Wanutan	-	wxwx Lk2Lk2 nn
Washotan	-	wxwx lk2lk2 NN
Washonutan	-	wxwx lk2lk2 nn

CHAPTER 3

RAPID DETERMINATION OF CEREAL ALPHA-AMYLASE USING AMYLOSE-AZURE BLUE PLATES

Introduction

To study the reported unique alpha-amylase characteristics of waxy, short-awn, hulless barleys, it was necessary to develop a rapid and simple assay for this enzyme that could be used to examine numerous samples of barley. The objectives of the research reported herein were to (1) compare the developed assay with established alpha-amylase assays, (2) determine the time/temperature profile of the assay for samples with widely different alpha-amylase activities, and (3) determine the assay's accuracy by following physiological events associated with alpha-amylase production.

Materials and Methods

General

The aforementioned gel-diffusion assay is based on chromogenic substrate (amylose-azure blue) suspended in an agar medium (Nobel Agar). Such assays have been described subsequent to the development of this method (Hejgaard and Gibbons, 1979).

The general principles behind such assays rely on the ability of alpha-amylase to diffuse into the agar and degrade the starch substrate. Upon degradation, the starch releases the attached dye and a zone of clearance is produced. Uniform wells are punched in the agar plates, and the enzyme extract or paste placed in the wells. The zone of clearance can be quantitatively determined, and the measurements compared to known alpha-amylase activity values.

For all of the individual experiments reported in this dissertation, the amylose-azure blue agar plates were made in the following manner. Amylose-azure blue (1.4 g, Cal Biochem) was added to 1000 ml of a 0.012 M $Ca(OAc)_2$, pH 5.3, solution along with 11 g of Noble agar. The mixture was autoclaved at 121 C for 20 minutes, cooled to 50 C and 40 ml poured into 150 mm x 15 mm disposable petri dishes. The plates were cooled and stored in a refrigerator.

Sample wells (36-44) were punched into the agar with a number 2 cork borer (5 mm in diameter). Grain samples (10-20 g) were ground in a Udy cyclone mill with a 0.5 mm screen. The flour samples were made into a paste by mixing 0.25 g flour with 0.5 ml of water. Each well was filled level full with a paste sample. In most of the studies, the plates were incubated 1 to 4 hours (depending on the amount of alpha-amylase present) at 65 C (unless otherwise specified).

After incubation, clear rings occurred around each well due to the alpha-amylase activity of the sample. The diameter of these zones of clearance was measured to the nearest 0.5 mm. The alpha-amylase was recorded as alpha-amylase area, which is the difference between the area of the zone of clearance and the area of the sample well (diameter = 5.0 mm, area = 19.63 mm). Hereafter, this assay will be called the Blue Plate Agar (BPA) assay.

Study I: The BPA Assay vs. the Cibacron Blue-Amylose Dye Release Assay

Flour samples from 11 pairs of Compana and Washonupana were used to compare the BPA assay with the Cibacron Blue-Amylose (CBA) dye release method (DeHaas et al., 1978). The paired barley cultivars were grown in many environments and included samples of sound (nonweathered, nonsprouted), weathered, sprouted, and heavily sprouted barley.

The alpha-amylase activity of each sample was measured once using the CBA method (see acknowledgements). The BPA assay was performed as described previously in this chapter using a randomized complete block design with three replications. Separate BPA plates were replications. The sample diameters were measured at 1 and 4 hours. F-tests were used to evaluate the effects of flour samples (treatments) and plates at each incubation period. The mean of the three replications of each

sample was used to calculate the correlation and regression coefficients between the BPA and CBA assays. The CBA dye release units were transformed to a $\log_{10}$ scale to obtain a linear relationship.

Study II: The BPA Assay vs. the Iodine Dextrin Color Loss Method

Nineteen barley samples, consisting of one sample each of 'Nupana', 'Wapana', 'Wanupana', 'Shopana', and 'Shonupana', and seven pairs of Compana and Washonupana, were used to compare the BPA Assay with the Iodine Dextrin Color Loss (IDC) method (MacGregor et al., 1971a). The barley was grown in many environments and included samples of sound, weathered, and badly sprout damaged barley seed.

The alpha-amylase activity of each sample was measured once using the IDC method (see acknowledgements). The BPA assay was performed as described in Study I of this chapter. The IDC units were transformed to a $\log_{10}$ scale to obtain a linear relationship between the BPA and IDC assays.

Study III: The BPA Assay vs. Degrees Lintner Reducing Power of Malt Extract

Wallerstein's Malt Amylase P. F. (1500° lintner) was used to compare the BPA Assay with the reducing power of a highly purified enzyme extract. Twenty-five hundred milligrams of the commercial enzyme was mixed with 1 ml of distilled water and thereafter diluted 1/10, 1/100,

1/1000, and 1/10,000. Each of the five treatments of known reducing power (375°L, 37.5°L, 3.75°L, 0.375°L, and 0.0375°L) was replicated four times in blocks across a single BPA plate. Since the samples were not in the form of a paste but rather a solution, it was necessary to micro-pipette each well to level full with the enzyme solution. A sample of commercial Compana malt was replicated twice on the plate as a check. The sample diameters were measured at 1 and 4 hours. F-tests were used to evaluate the effect of the enzyme extract treatments and the within plate variability at each of the incubation periods. The mean of the four replications of each treatment was used to calculate the correlation and regression coefficients between the BPA assay and reducing power. Reducing power units (°L) were transformed to a $\log_{10}$ scale to obtain a linear relationship.

Study IV: Among Plate Variability of the BPA Assay

Four samples of Washonupana with widely different alpha-amylase activities, ranging from sound seed to very badly sprouted seed (50%) were selected from the samples used in Study I. These samples were used as checks on BPA plates run in unrelated experiments over a period of months. Single determinations of alpha-amylase area for these four samples were selected from six plates to determine the plate-to-plate variability over time. The sample diameters were

measured at 4 hours only. An F-test was used to evaluate the effect of samples and plates. All six replications of each sample were used to calculate the correlation and regression coefficients between the BPA assay and the CBA assay.

Study V: The Effect of Time and Temperature on the Ability of the BPA Assay to Measure Alpha-Amylase

The four samples of Washonupana barley with widely different levels of alpha-amylase activity which were used in Studies I and IV of this chapter and a sample of Klages malt from Study VIII were used to examine the effect of time and temperature on the ability of the BPA assay to measure alpha-amylase activity. Each sample was replicated two times on each BPA plate. Individual plates were incubated at 25, 35, 45, 55, 65, or 75 C. Sample diameters were measured at 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 hours. F-tests were performed to evaluate the effects of samples, incubation times and incubation temperatures. For each temperature regimen, the mean alpha-amylase area of the two replications was used to plot the relationship between alpha-amylase area and incubation time.

Study VI: Alpha-Amylase Activity Measured by the BPA Assay vs. Germination Time

'Shabet' and 'Klages' barley samples were used to compare alpha-amylase activity with germination time using the BPA assay. Both

cultivars were grown at Huntley, Montana (MT) in 1978. Ten grams of seed of each cultivar were placed between moistened blotters within a 125 x 135 mm germination box (sandwich box). Twelve boxes of each cultivar were placed in a germination chamber at 15 C. At 24, 48, 72, and 96 hours, three boxes of each cultivar were removed from the germination chamber and kilned for 24 hours at 65 C. The dried samples and a nongerminated sample of each cultivar were ground to flour in a Udy Cyclone Mill (0.5 mm screen). Each of the three germination replications for each germination interval (24, 48, 72, and 96 hours) was replicated on three separate BPA plates. The ungerminated sample was also replicated on the three separate BPA plates. All samples were randomized on each plate. The sample diameters were measured after 1 hour. The comparison between alpha-amylase area (BPA assay) and germination time was plotted using the mean of three samples for the ungerminated treatment and the mean of nine samples for each of the germination intervals (24, 48, 72, and 96 hours).

Study VII: Alpha-Amylase Activity Measured by BPA Assay vs. Percent Sprouted Seed

Individual sprouted seed of Shabet and Klages were saved from Study VI to compare alpha-amylase activity as measured by the BPA assay with the percent sprouted seed in a sample. The seeds used were taken from the 48 hour germination samples which had seminal

roots 3-5 mm long. The sprouted kilned seeds were added to non-germinated seeds in the following proportions: 0/100, 1/100, 2/100, 3/100, 4/100, 5/100, and 6/100. Each treatment was replicated three times. The seed samples were ground to flour in a Udy Cyclone mill. The 21 flour samples were randomized on a single BPA plate. Sample diameters were measured at 1 hour. The mean of the three replications of each treatment was used to calculate the correlation and regression coefficients between the BPA assay and percent sprouted seed.

Study VIII: The Use of the BPA Assay in Distinguishing Between the Malt Alpha-Amylase Activity of Feed and Malting Barley

The feed barleys, Titan and Compana, the malting barley, Betzes, and the high-enzyme malting barley, Klages, were grown as replicated (3) single row entries in eight Montana environments (see Chapter 6 for specific environments). The cultivar Betzes is the recurrent parent for Shabet, thus, the two cultivars are similar. Each sample was micromalted (see Chapter 5) and the alpha-amylase activity of the finished malt was determined using the BPA assay (1 hour incubation). The activity measured as alpha-amylase area was converted to the equivalent CBA dye release units.

Results and Discussion

General

The alpha-amylase assay described in this paper is similar to an assay described by Hejgaard and Gibbons (1979). The main differences are that this assay requires no extraction, the results are obtained quicker, and alpha-amylase activity is measured as an area rather than a diameter.

The 65 C incubation temperature was originally chosen since beta-amylase is inactivated within 20 minutes at this temperature. The substrate used in this assay, amylose-azure blue, is reported to be unaffected by beta-amylase (Klein et al., 1969). However, those reports are based on samples in which at least a crude extraction of alpha-amylase has been prepared. In this study, no attempt was made to separate enzymes. Thus, as an added precaution, the 65 C temperature was used. At this temperature alpha-amylase is active but not at its peak activity (50-55 C optimum) (Steiner, 1976).

For sound samples of barley grain, the term paste is a fairly accurate description of the flour/water suspension placed into the sample wells. However, in the case of the highly modified material such as malt, the flour/water suspension is grist like and must be souped into the wells in a very liquid form.

A BPA plate with samples and the resultant digestion diameters/areas is shown in Figure 3-1. The diameter of these zones of clearance is not an accurate measurement of such a zone. A 1 mm ring, 5 mm from the sample well, has an area 2.5 times greater than the area of a 1 mm ring immediately next to the well. While the final activity is measured as an area, the precision of the assay depends upon how precisely diameters can be measured. Experience indicates that measurements can be made precisely to within 0.5 mm. Others, using similar assays (Hejgaard and Gibbons, 1979), feel that measurements can be made to within 0.25 mm.

Study I: The BPA Assay vs. Cibarcon Blue-Amylose Dye Release Assay

Highly significant correlations were obtained between the BPA and CBA assays after 1 ($r = .97^{**}$) and 4 ($r = .99^{**}$) hours of incubation (Figures 3-2 and 3-3). The two assays use similar substrates to measure alpha-amylase activity. Thus, a correlation between the two was expected. The high correlation indicates that the faster BPA assay could be substituted for the CBA assay.

Significant differences among flour samples were detected at both incubation times (Table 3-1 and 3-2). This was expected since the samples ranged from sound to heavily sprouted barley seed. The among-plate variation was nonsignificant. For the 1 hour incubation time,

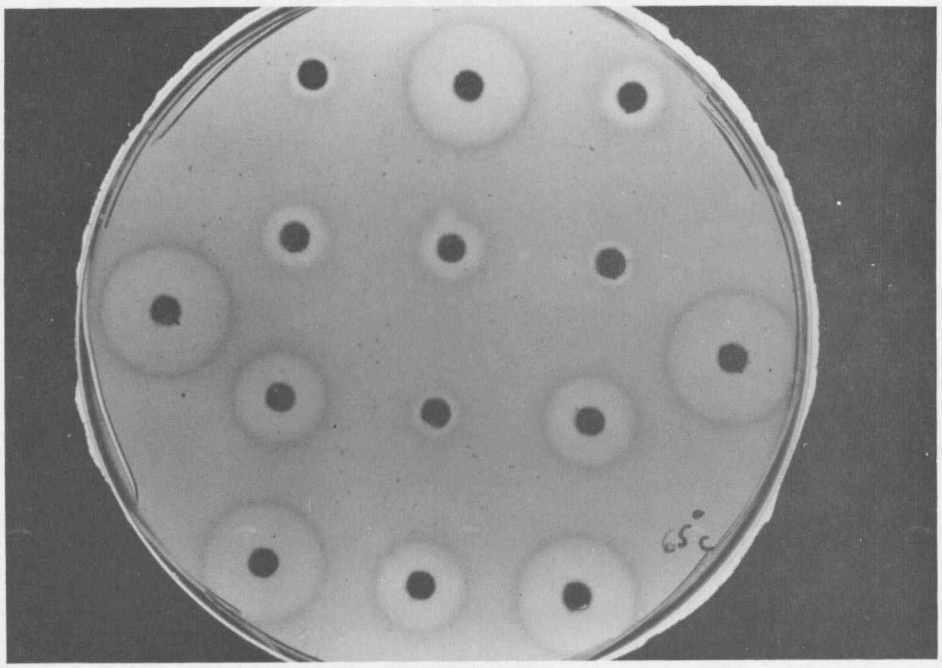

Figure 3-1. A BPA assay plate (150 mm x 15 mm) with 14 samples ranging from sound seed to Klages malt and the resulting digestion diameters/ areas after 4 hours of incubation at 65 C.

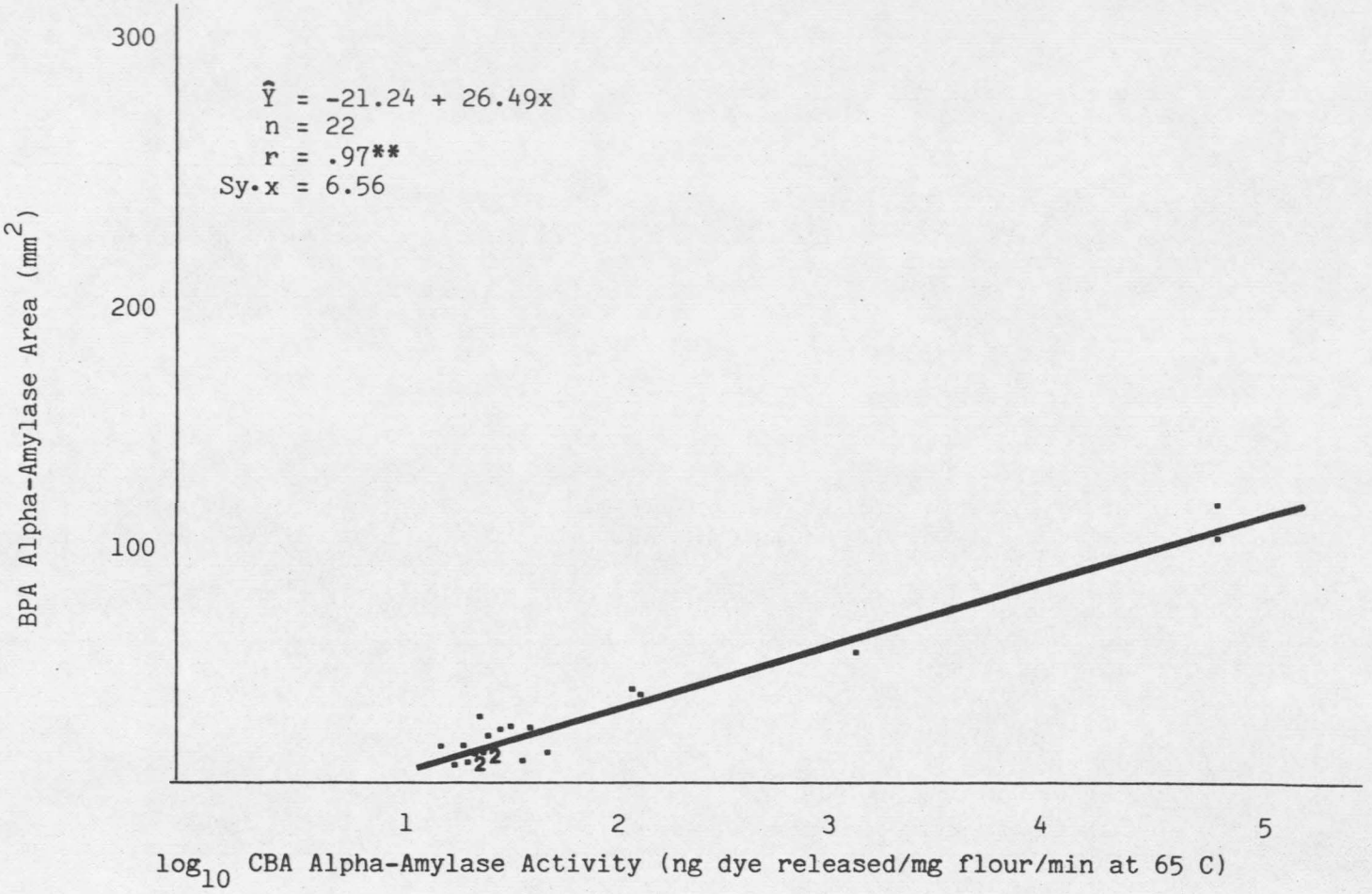

Figure 3-2. Regression of BPA alpha-amylase area at 1 hour of incubation to CBA alpha-amylase activity for 22 barley flour samples.
**Significant at the 0.01 level.

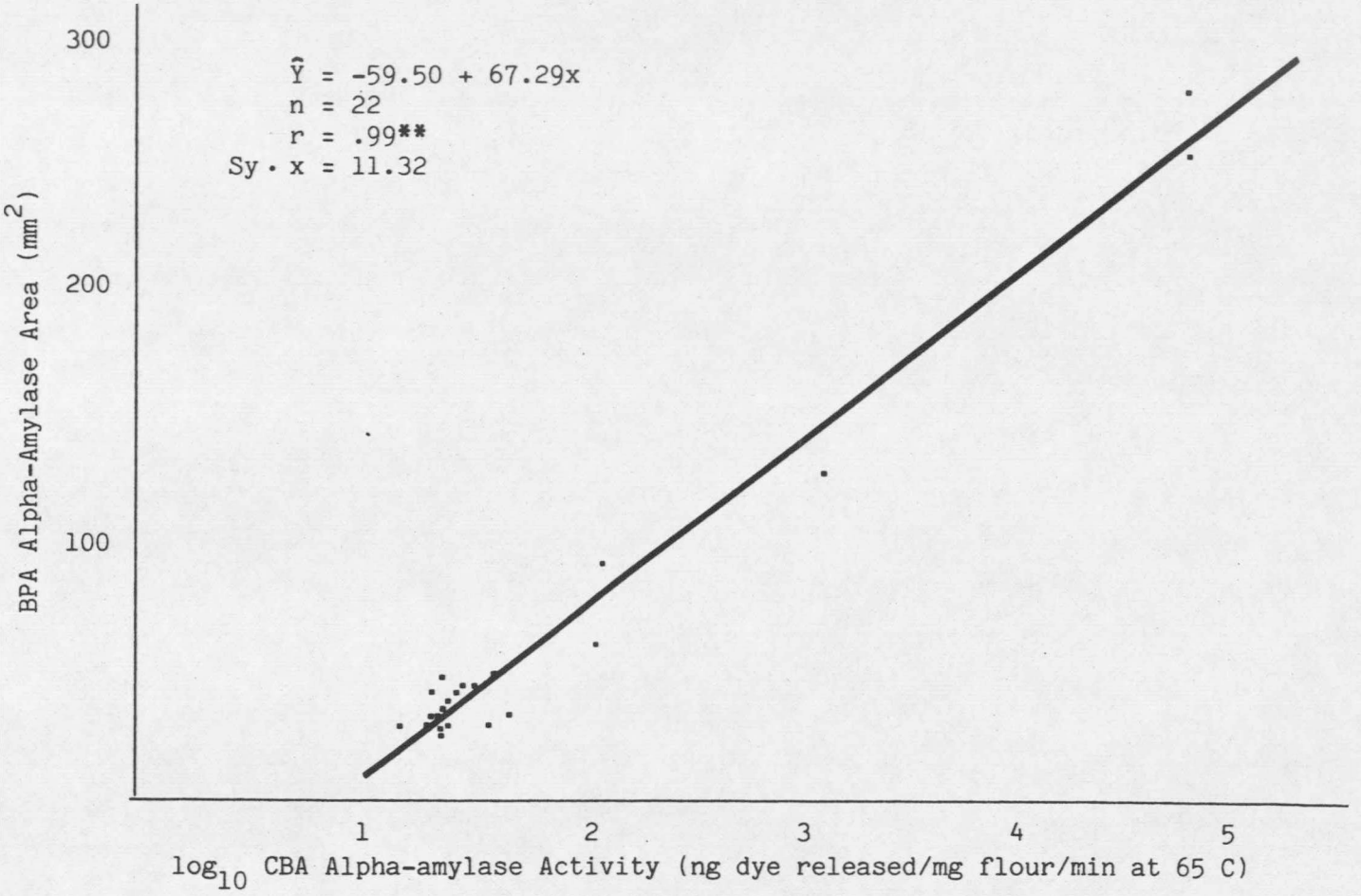

Figure 3-3. Regression of BPA alpha-amylase area at 4 hours of incubation to CBA alpha-amylase activity for 22 barley flour samples.
**Significant at the 0.01 level.

Table 3-1. Analysis of variance and coefficient of variation (C.V.) for BPA alpha-amylase area (mm^2) of 22 barley samples at 1 hour of incubation.

Source of Variance	df	SS	MS	F
Barley Samples	21	48445.07	2306.90	64.46**
Plates	2	0.96	0.48	0.01
Error	42	1503.26	35.79	
C.V. % = 23.0				

**Significant at the 0.01 level.

Table 3-2. Analysis of variance and coefficient of variation (C.V.) for BPA alpha-amylase area (mm^2) of 22 barley samples at 4 hours of incubation.

Source of Variance	df	SS	MS	F
Barley Samples	21	311752.13	14845.30	1067.24**
Plates	2	29.15	14.58	1.05
Error	42	584.22	13.91	
C.V. % = 6.2				

**Significant at the 0.01 level.

the F-value associated with the among-plate variation was very low (0.01). When the mean square (MS) for plates was used as the denominator and the MS for error (plate x treatment interaction) used as the numberator, the resulting F-value was significant (P = .01), indicating a significant plate x treatment interaction.

The cause of this interaction was probably because treatments were randomized within plates. Some of the plastic disposable plates are bowed in the middle, thus, the thickness of the agar is not uniform across the entire plate. This problem can be alleviated by using glass plates. The small size of the 1 hour digestion diameters might also be a factor. They were more difficult to measure precisely. This was reflected in a high coefficient of variation (23.0%) at 1 hour of incubation.

The coefficient of variation (C.V.) of the 4 hour incubation time was 6.2%. The lower C.V. observed in the 4 hour incubation compared to the 1 hour incubation was probably due to increased measurement precision of the larger average digestion areas. The size of the digestion diameter increased considerably between 1 hour and 4 hours of incubation at 65 C, and at the same time, the mean square error was reduced. Thus, the C.V. was reduced (C.V. = MSE/ y).

Study II: The BPA Assay vs. the Iodine Dextrin Color Loss Method

Highly significant correlations were obtained between the BPA and IDC assay after 1 ($r = .88^{**}$) and 4 ($r = .89^{**}$) hours of incubation (Figures 3-4 and 3-5). These correlations were not as high as those obtained with the BPA vs. CBA assays. Three samples showed no alpha-amylase by the IDC method, while enzyme activity was detected with the BPA and CBA methods. This indicates that the BPA assay was more sensitive than the IDC assay, and may account for the lower correlation coefficients.

Significant differences among samples were detected at both incubation times (Table 3-3 and 3-4). This was expected, since the samples ranged from sound to badly sprouted barley seed. No significant plate-to-plate variation was observed. As in the previous study, the C.V.'s decreased with incubation time.

Study III: The BPA Assay vs. Degrees Lintner Reducing Power of Malt Extract

Highly significant correlations were obtained between the BPA assay and the activity of the malt extract dilution series at 1 ($r = .98^{**}$) and 4 ($r = .99^{**}$) hours of incubation (Figures 3-6 and 3-7).

Significant differences among extract dilutions were detected at both incubation times (Tables 3-5 and 3-6). The within-plate variability was minimal, and in the case of the 1 hour incubation,

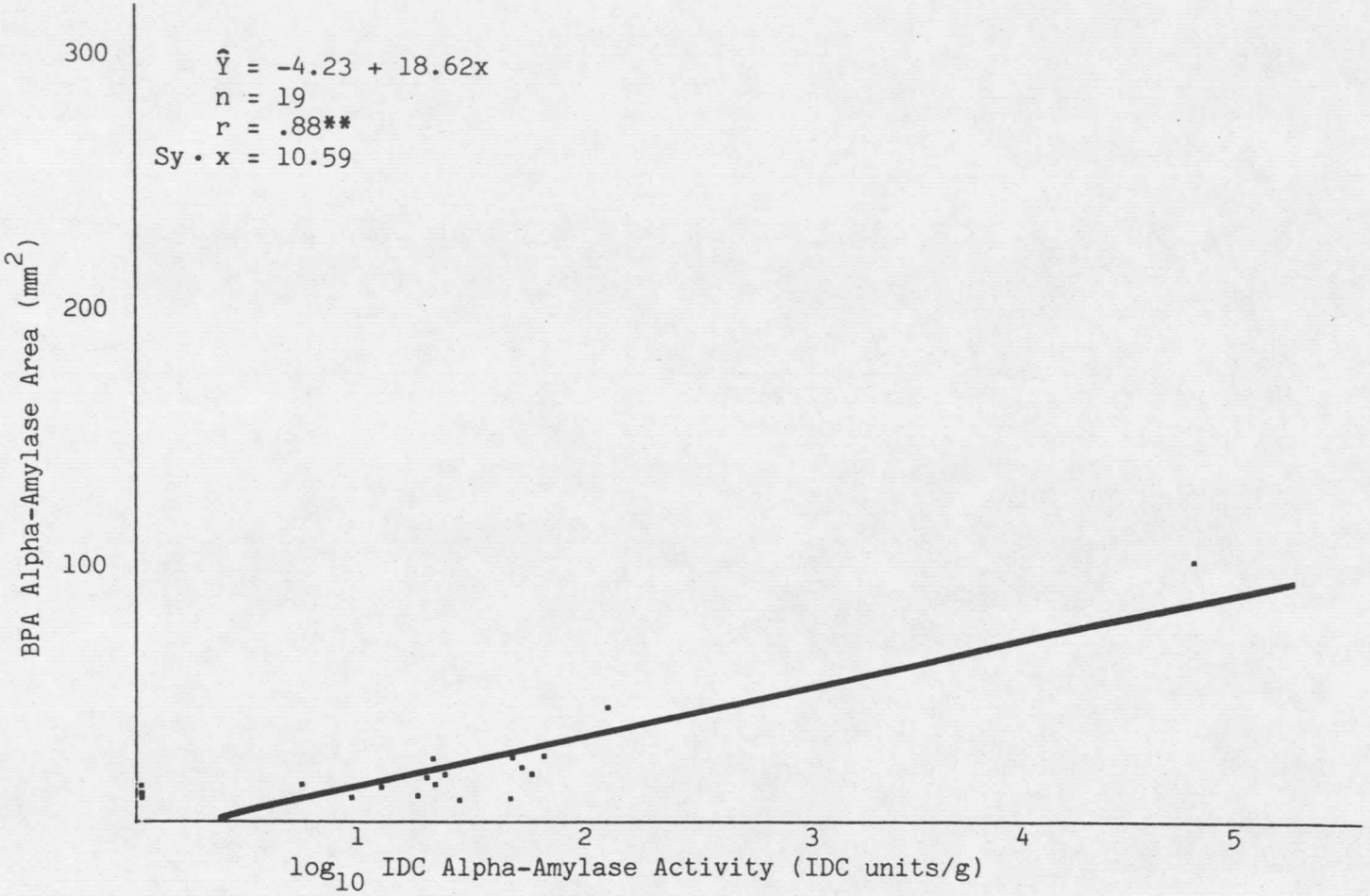

Figure 3-4. Regression of BPA alpha-amylase area at 1 hour of incubation to IDC alpha-amylase activity for 19 barley flour samples.
**Significant at the 0.01 level.

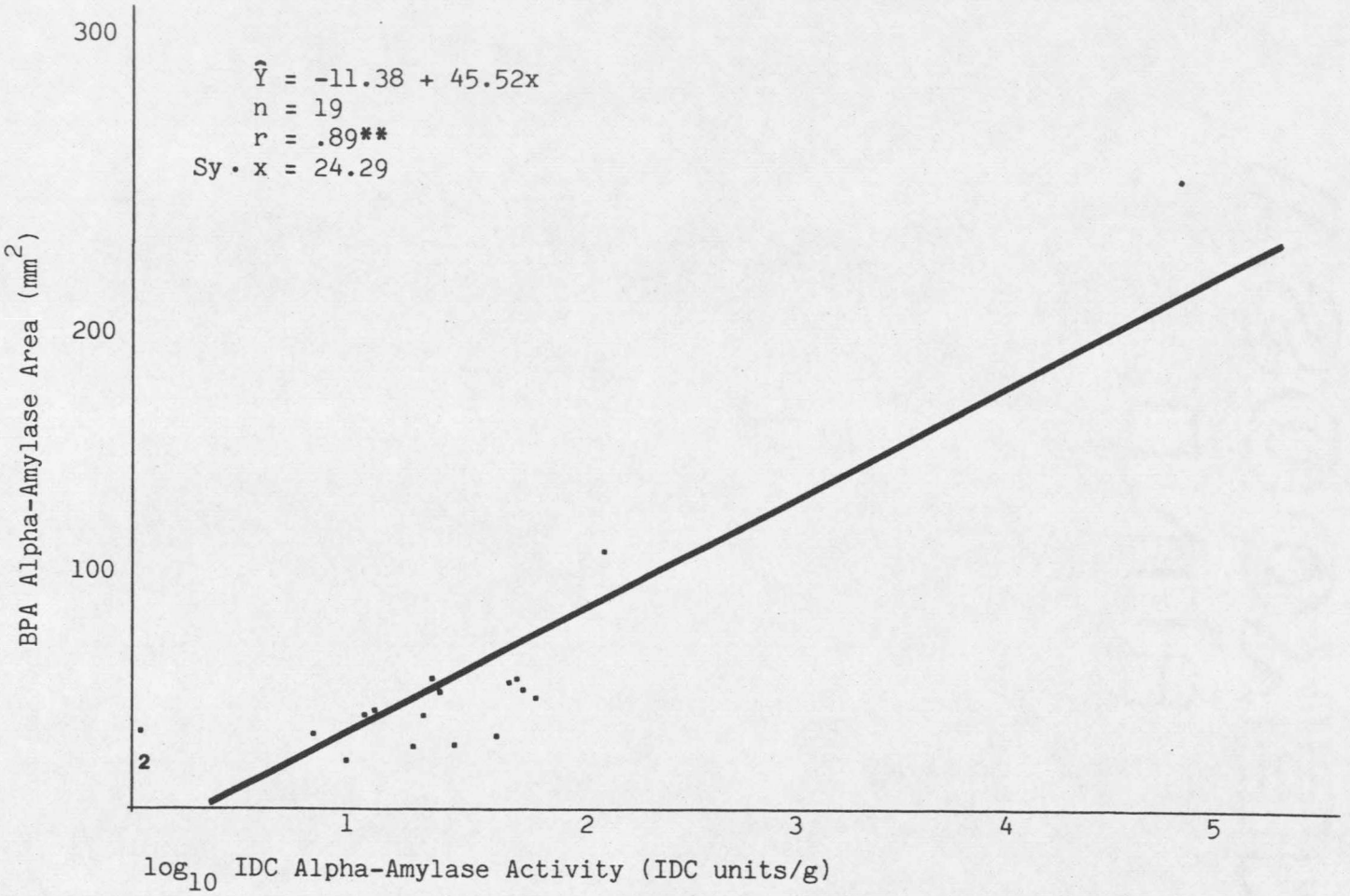

Figure 3-5. Regression of BPA alpha-amylase area at 4 hours of incubation to IDC alpha-amylase activity for 19 barley flour samples.
**Significant at the 0.01 level.

Table 3-3. Analysis of variance and coefficient of variation (C.V.) for BPA alpha-amylase area (mm^2) of 19 barley samples at 1 hour of incubation

Source of Variance	df	SS	MS	F
Barley Samples	18	25700.00	1427.80	302.50**
Plates	2	2.59	1.30	0.28
Error	36	170.06	4.72	
C.V. % = 10.2				

**Significant at the 0.01 level.

Table 3-4. Analysis of variance and coefficient of variation (C.V.) for BPA alpha-amylase area (mm^2) of 19 barley samples at 4 hours of incubation.

Source of Variation	df	SS	MS	F
Barley Samples	18	149710.00	8316.00	1100.00**
Plates	2	16.38	8.19	1.08
Error	36	272.11	7.56	
C.V. % = 5.4				

**Significant at the 0.01 level.

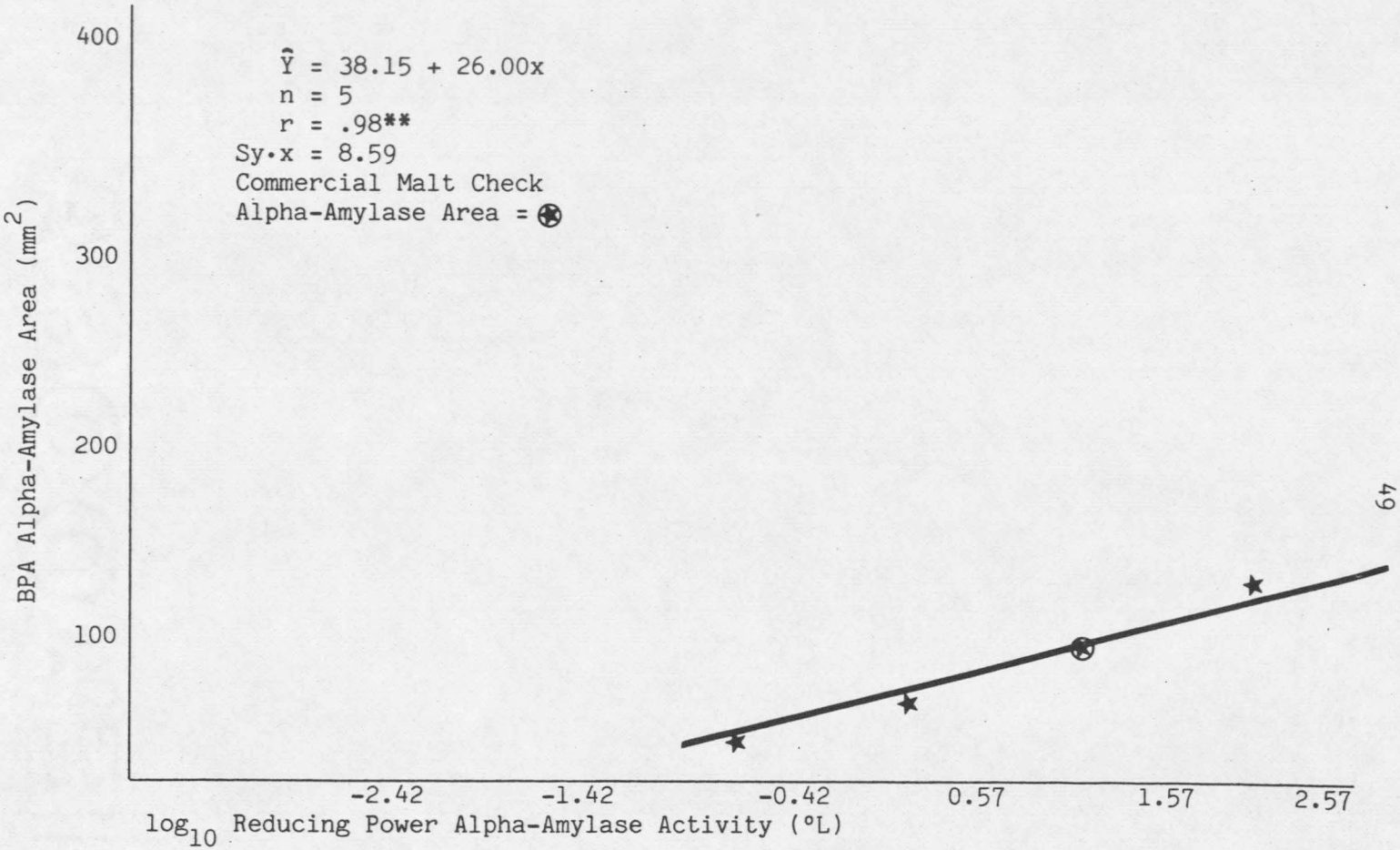

Figure 3-6. Regression of BPA alpha-amylase area at 1 hour of incubation to reducing power alpha-amylase activity for five malt extract dilutions.
**Significant at th 0.01 level.

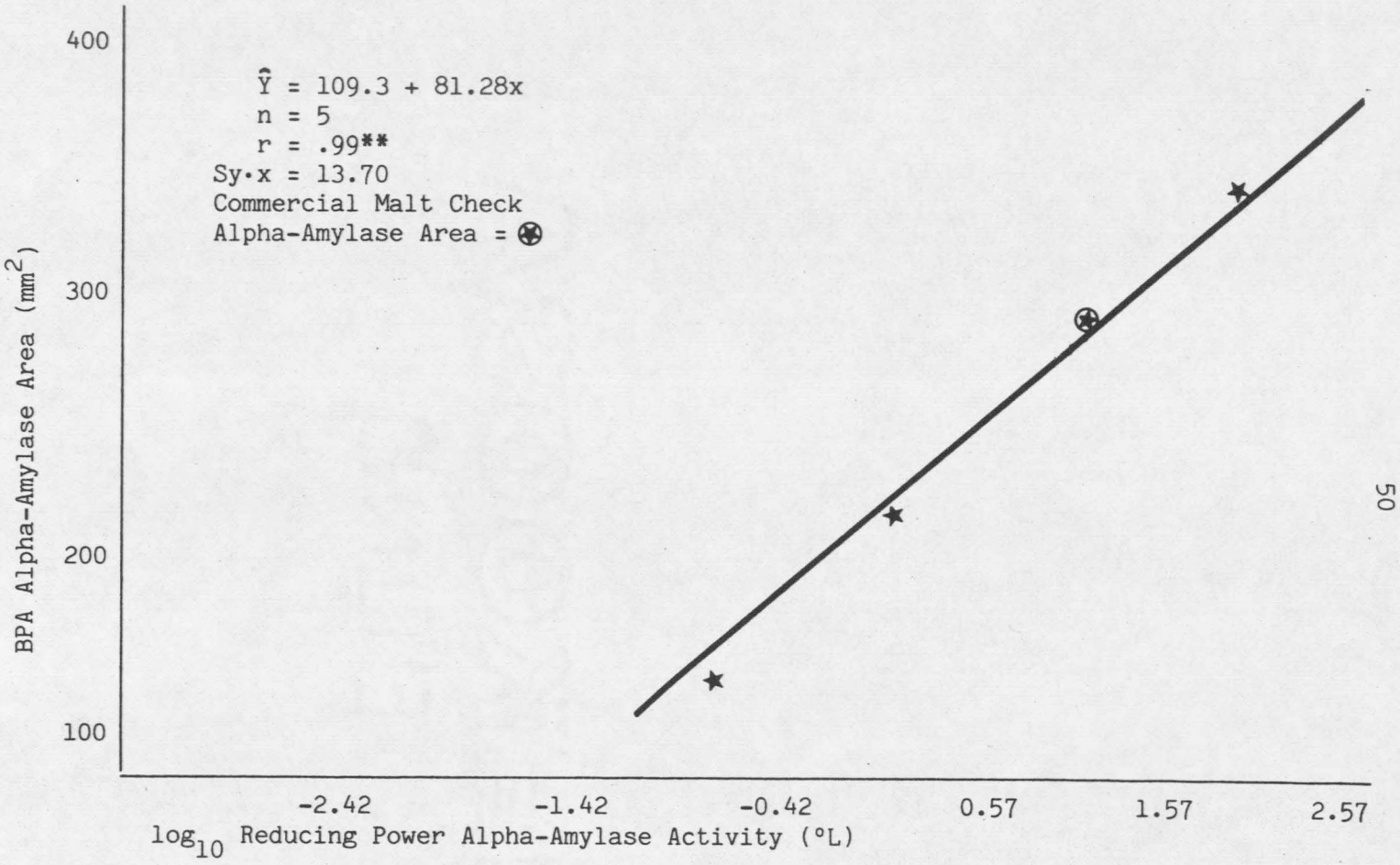

Figure 3-7. Regression of BPA alpha-amylase area at 4 hours of incubation to reducing power alpha-amylase activity for five malt extract dilutions.
**Significant at the 0.01 level.

Table 3-5. Analysis of variance and coefficient of variation (C.V.) for BPA alpha-amylase area (mm^2) of five malt extract dilutions at 1 hour of incubation.

Source of Variance	df	SS	MS	F
Malt Extract	4	27865.21	6966.30	-
Replications	3	0	0	-
Error	12	0	0	
C.V. % = 0				

Table 3-6. Analysis of variance and coefficient of variation (C.V.) for BPA alpha-amylase area (mm^2) of five malt extract dilutions at 4 hours of incubation.

Source of Variance	df	SS	MS	F
Malt Extract	4	265850.39	66462.60	9934.62**
Replications	3	26.77	8.92	1.33
Error	12	80.32	6.69	
C.V. % = 1.66				

**Significant at the 0.01 level.

it was 0 (relative to measuring technique). In general, it has been noted that for the better plastic plates (little bowing), within-plate variation was negligible. However, this was the only study in this series of experiments designed to show this statistically.

The alpha-amylase area of the commercial malt was identical to the malt extract dilution equivalent to 37.5 °L (⊛ in Figures 3-6 and 3-7). The most active malt extract sample (375 °L) was more active than the commercial malt indicating that the assay tests over a broad range of alpha-amylase activity.

Study IV: Among Plate Variability of the BPA Assay

The correlation between the BPA values and the CBA values of the four check samples was r = .99** (Figure 3-8). The individual data points about a given sample are remarkably uniform considering these BPA assays were performed by a number of workers over a period of time. The F-test (Table 3-7) for plate effect was nonsignificant while, the sample activity effect was highly significant. These results indicated that the four check samples behaved similarly on different plates when incubated at the same temperature and for the same period of time.

As a result of the excellent correlation between the CBA method and the BPA assay, the four samples used in this study were used in

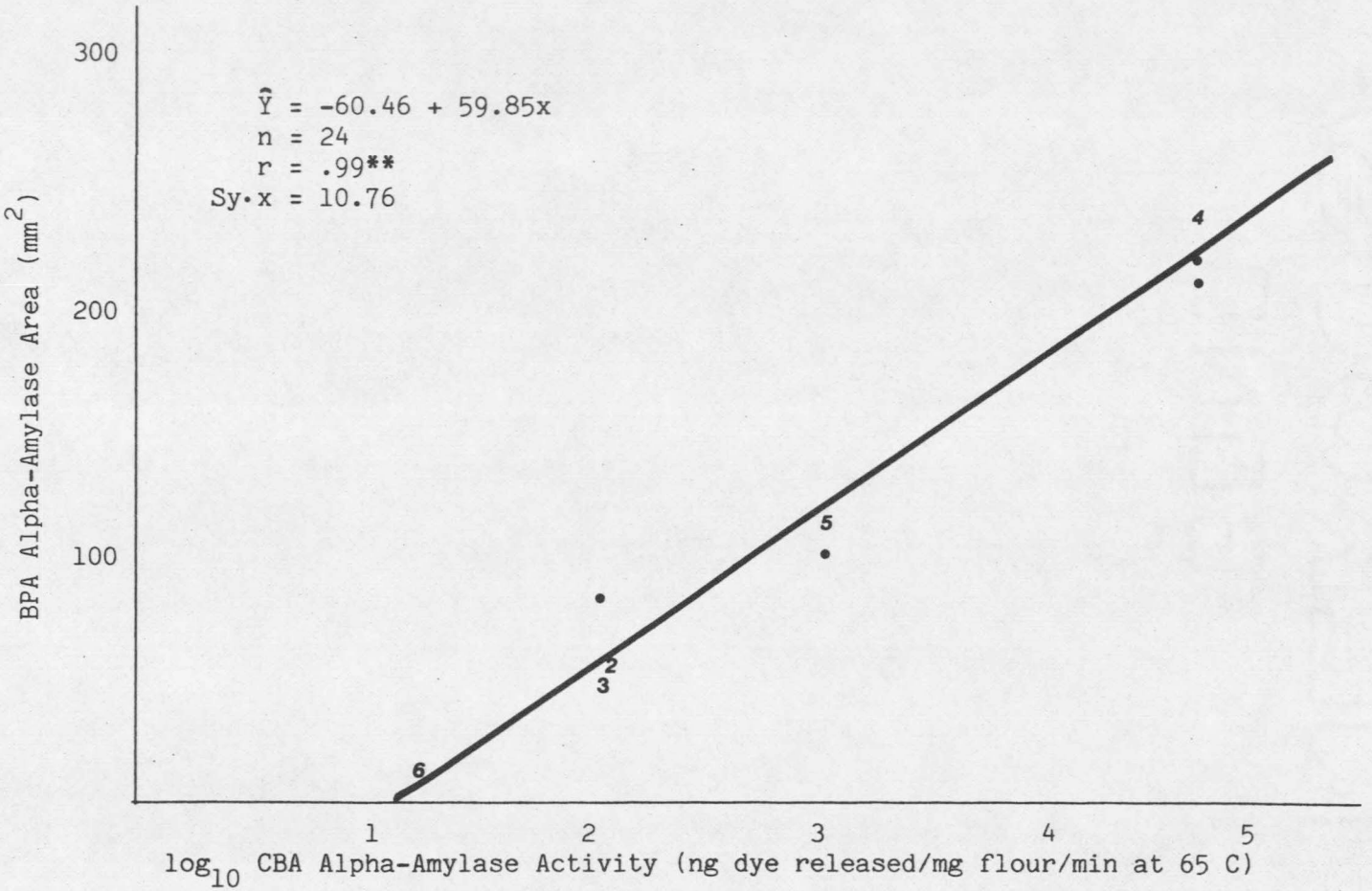

Figure 3-8. Regression of BPA alpha-amylase at 4 hours of incubation to CBA alpha-amylase activity for four standard barley flour samples.
**Significant at the 0.01 level.

Table 3-7. Analysis of variance and coefficient of variation (C.V.) for BPA alpha-amylase area (mm^2) of four standard samples at 4 hours of incubation.

Source of Variance	df	SS	MS	F
Standard Samples	3	148600.00	49520.00	750.42**
Plates	5	207.50	41.51	0.63
Error	15	989.80	65.99	
C.V. % = 7.8				

**Significant at the 0.01 level.

subsequent studies to standardize the alpha-amylase activity detected on BPA plates. The four samples were included on all BPA plates as checks. A regression equation relating alpha-amylase area to CBA dye release units was calculated from these checks and used to convert all the BPA area values into dye release units for each plate. Thus, any bias associated with individual plates can be removed. Also, the activities of samples on plates incubated for different lengths of time can be converted to directly comparable units. Finally, the alpha-amylase area unit is not one that is directly related to alpha-amylase activity. Rather, it is proportional to the $\log_{10}$ of enzyme activity. By converting the BPA data to CBA dye release units, the alpha-amylase activity is reported in units directly related to actual activity.

Study V: The Effect of Time and Temperature on the Ability of the BPA Assay to Measure Alpha-Amylase

The purpose of this study was not to provide an indepth enzyme kinetic study but rather to provide the researcher a guide as to what time/temperature regimen might best suit the research needs.

Significant differences in alpha-amylase area were detected among all main effects, temperature, time, and samples (Table 3-8). The difference among samples was expected since five barley flour samples were chosen that had different levels of activity. Time and

Table 3-8. Analysis of variance of BPA alpha-amylase area (mm^2) for five selected samples at six temperatures and ten incubation times.

Source of Variance	df	SS	MS	F
Temperature (Te)	5	1241000.00	248200.00	27455.75**
Time (Ti)	9	1234000.00	137111.11	15167.16**
Samples (S)	4	3708000.00	927000.00	102544.25**
Te x Ti	45	256100.00	5690.00	629.42**
Te x S	20	313900.00	15691.11	1735.74**
Ti x S	36	614700.00	17075.00	1888.83**
Te x Ti x S	180	86500.00	480.55	53.16**
Error	300	2711.29	9.04	

**Significant at the 0.01 level.

temperature were known to influence enzyme activity. All interactions were significant, but none affected the rank order of the samples at any time or temperature treatment.

The $LSD_{.01}$ for differences between flour samples measured as alpha-amylase area was 0.997 mm^2. No individual using a ruler could detect such a small difference. This low value is a result of the majority of duplicate readings being identical. Those that did vary, usually differed by 0.5 mm (diameter). This was true for all readings, regardless of samples, time, or temperature. The detectable differences were .25-.50 mm for diameter and 5-15 mm^2 for area. The five flour samples differed by at least these latter parameters for all time/temperature combinations.

At the 25 C incubation temperature (Figure 3-9), it took 7 hours to obtain a reaction from the lowest activity sample. The three lowest activity samples behaved similarly with unevenly increasing alpha-amylase activity with incubation time. There was just minimum differentiation (alpha-amylase area) among samples. The two high activity samples were well differentiated and activity increased evenly with time.

At 35 C (Figure 3-10), it took 5 hours for the lowest activity sample to give a reaction. The general shape of all five activity vs. time curves was more symmetrical, marked by a steady increase

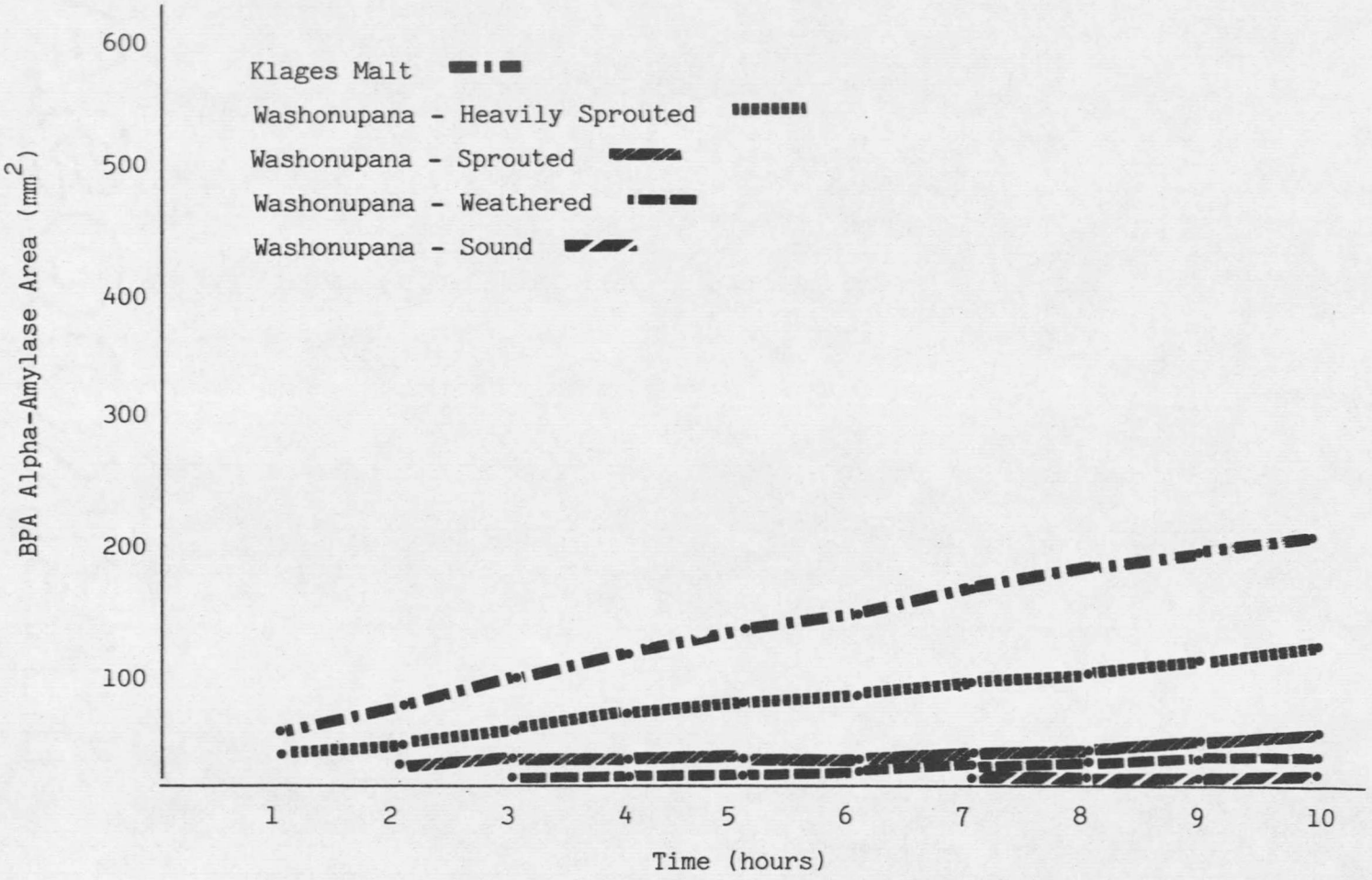

Figure 3-9. BPA alpha-amylase area of five barley flour samples incubated at 25 C for 1-10 hours.

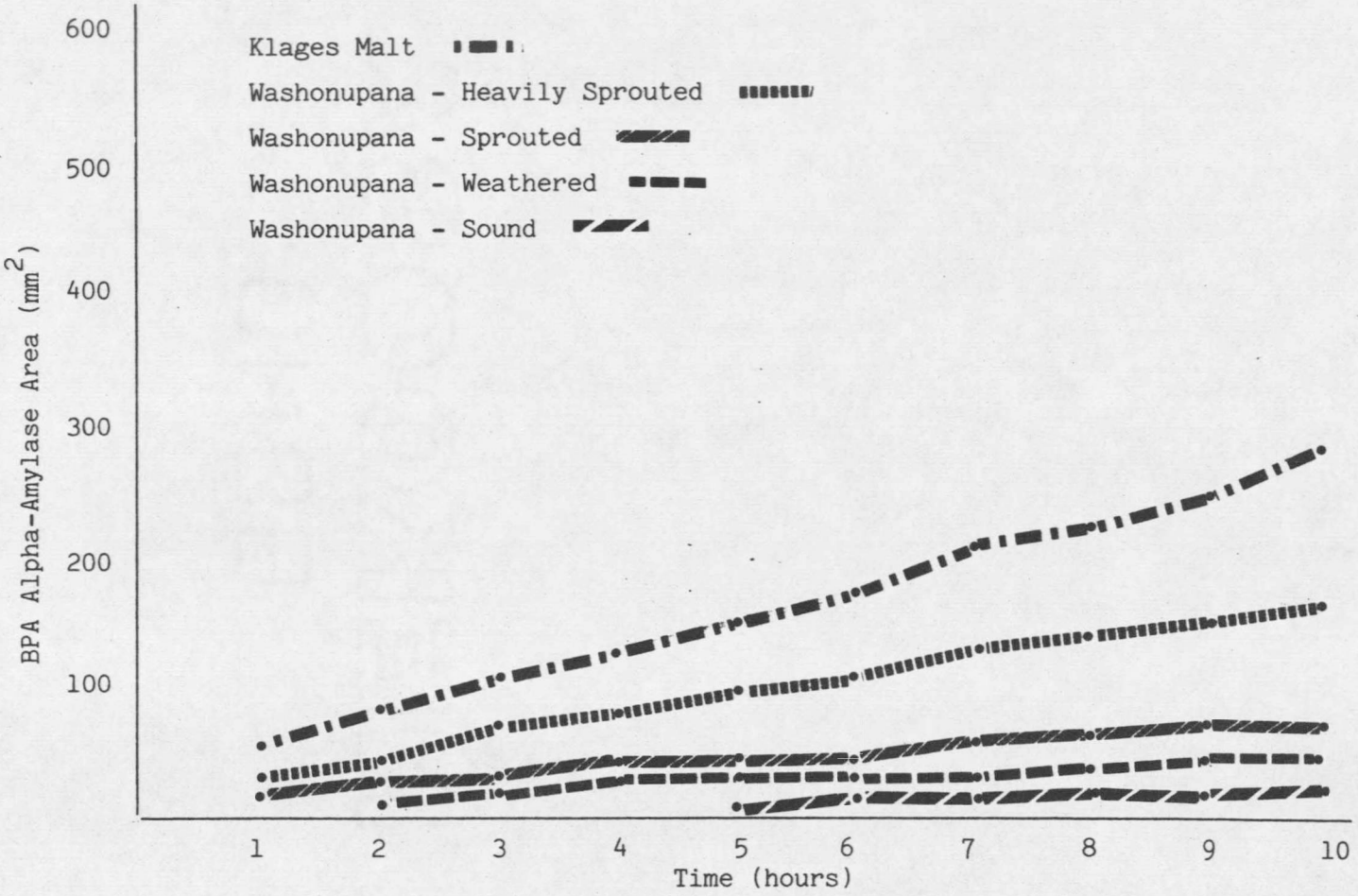

Figure 3-10. BPA alpha-amylase area of five barley flour samples incubated at 35 C for 1-10 hours.

in alpha-amylase area with time. The differential between samples increased at this temperature.

At 45 C (Figure 3-11), the low activity sample could be read at 3 hours and all five samples had a consistent rise in alpha-amylase area with incubation time. The alpha-amylase area differential between the three low samples increased greatly.

At 55 C (Figure 3-12), all samples could be read at the 1 hour incubation and all samples produced linear increases of area with time. Maximum alpha-amylase areas were observed for the three low samples. This temperature resulted in the best differential between the three lowest samples.

At 65 C (Figure 3-13), the three lowest samples had reduced alpha-amylase areas but still produced good reactions with good differential between samples. For the two high-activity samples, this proved to be the optimum temperature for alpha-amylase area development.

At 75 C (Figure 3-14), alpha-amylase was severely inhibited and all five samples had reduced alpha-amylase area development. It took four hours before the lowest sample could be read. The differential among the low three samples was greatly reduced. This is an unusable temperature for this assay because the agar is almost liquid at this temperature.

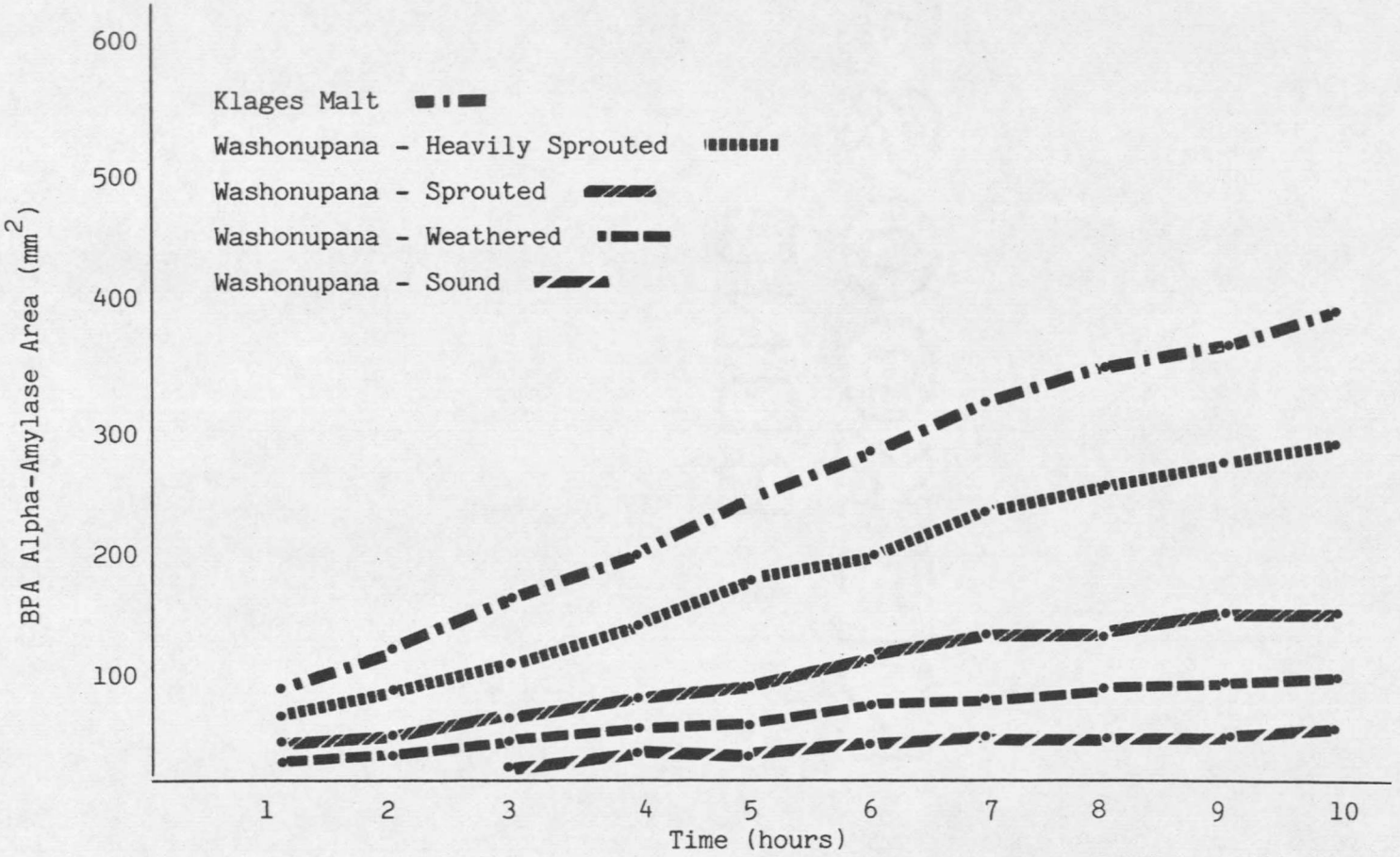

Figure 3-11. BPA alpha-amylase area of five barley flour samples incubated at 45 C for 1-10 hours.

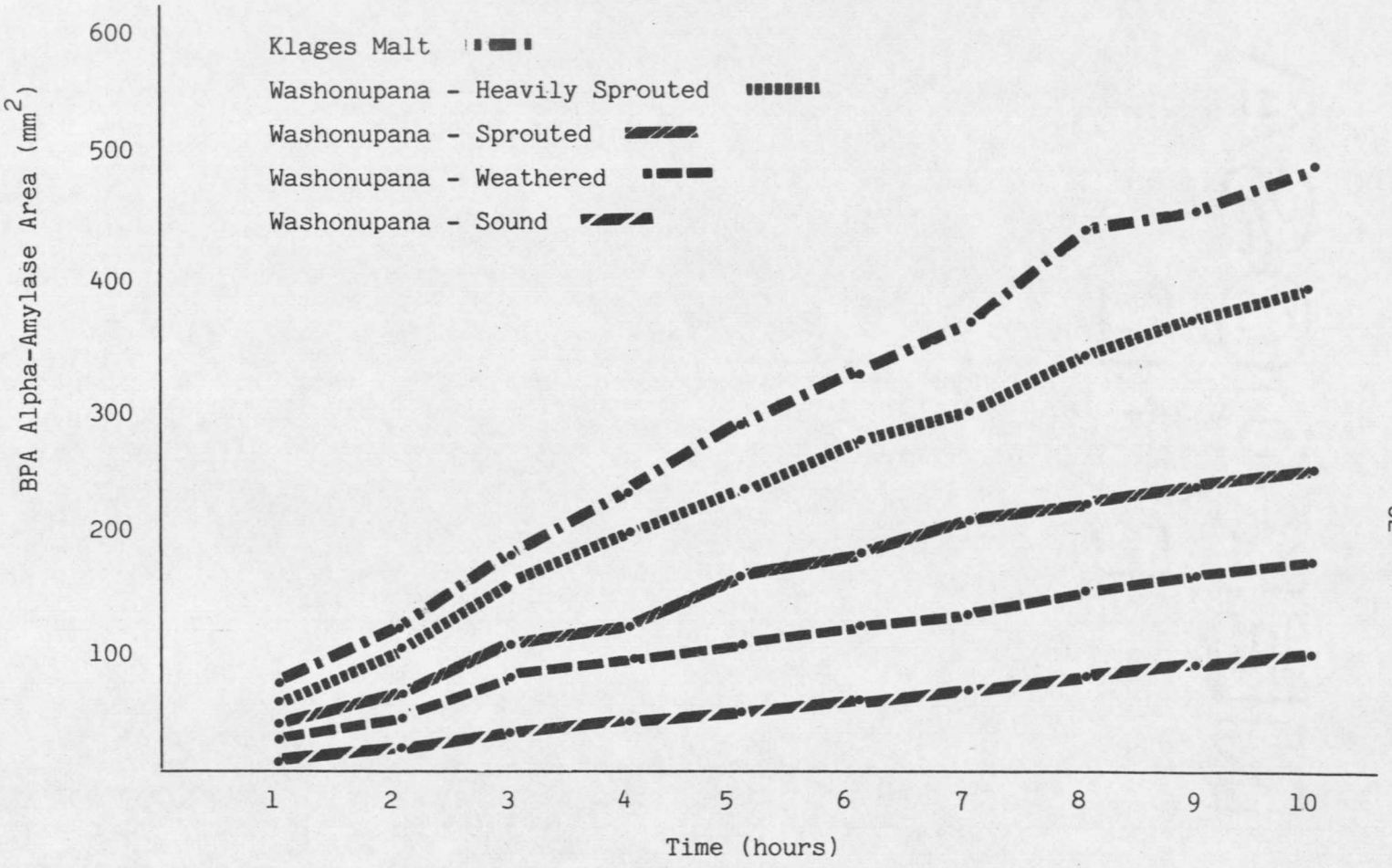

Figure 3-12. BPA alpha-amylase area of five barley flour samples incubated at 55 C for 1-10 hours.

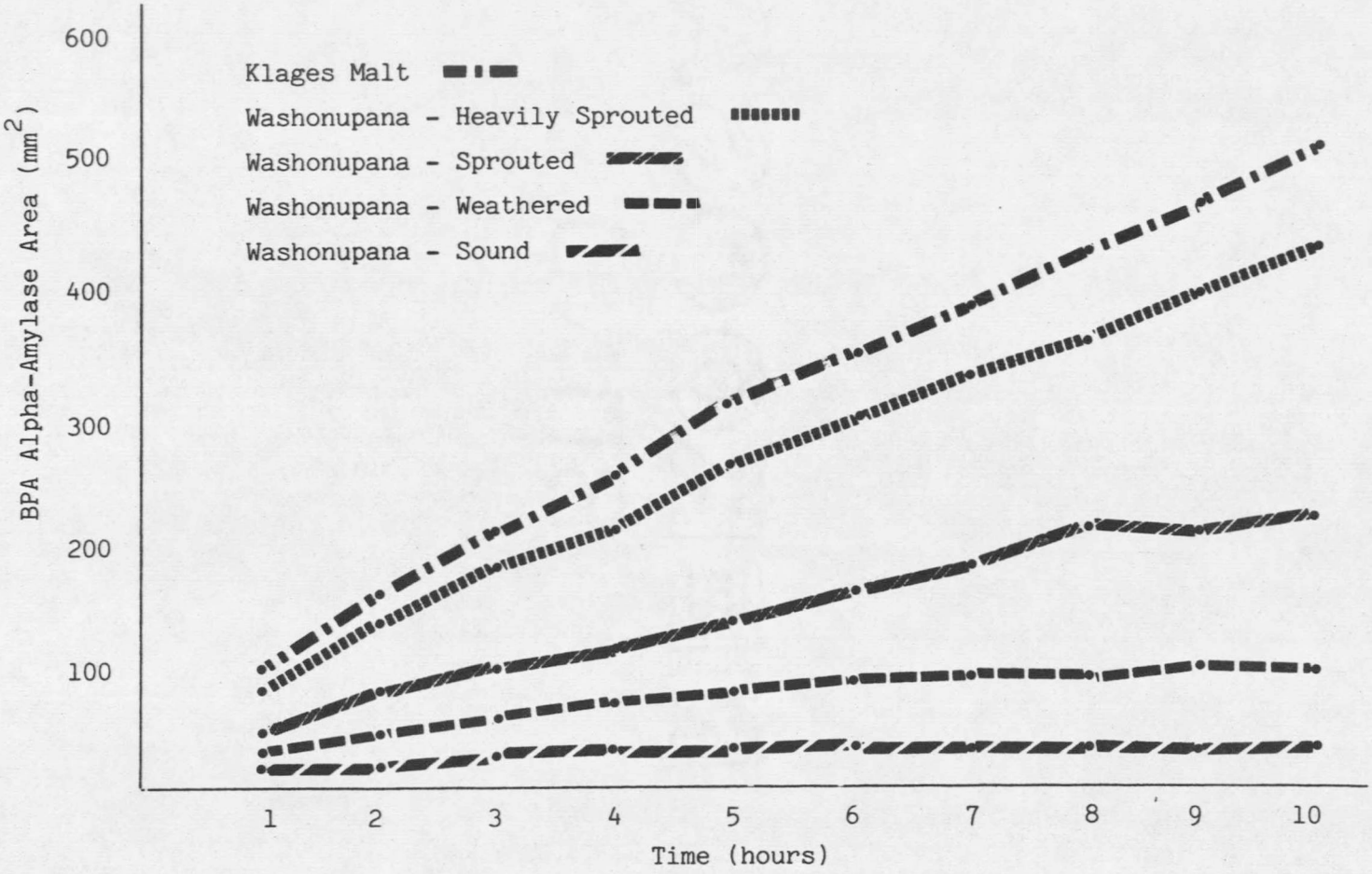

Figure 3-13. BPA alpha-amylase area of five barley flour samples incubated at 65 C for 1-10 hours.

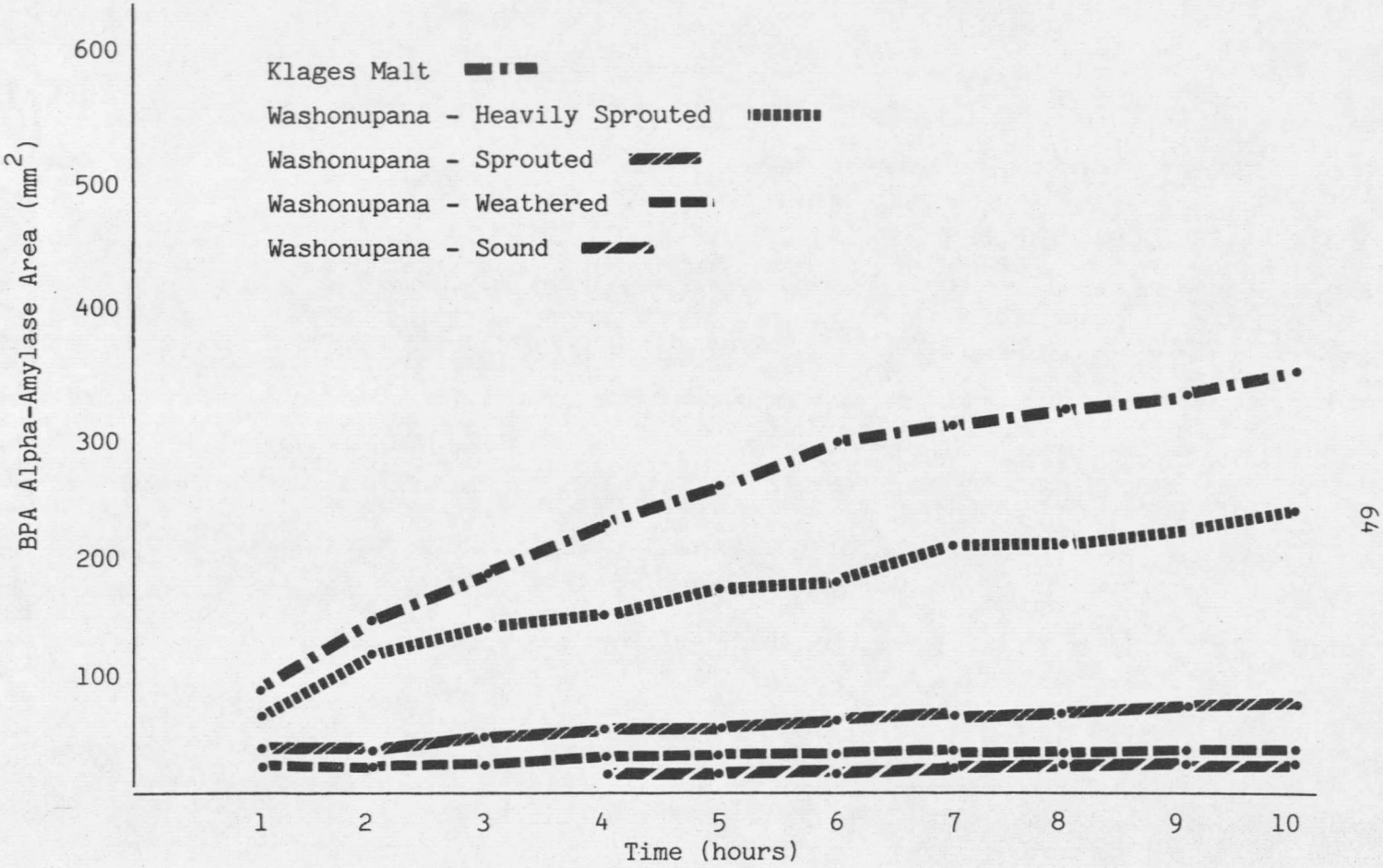

Figure 3-14. BPA alpha-amylase area of five barley flour samples incubated at 75 C for 1-10 hours.

Good results were obtained with high activity samples (malt and very heavy sprout damage) at any temperature between 25-65 C. Peak digestion areas occurred at 65 C for both high samples, but peak differential between the two samples occurred at 35 C.

The best results for the low activity samples were obtained at 55 C. Maximum alpha-amylase areas and maximum differential among samples was obtained at this temperature. Thus, for lower activity samples, and for a wide range of samples, 55 would probably be the best incubation temperature.

The differential between all samples increased with time up to 10 hours. As a balance between efficiency and precision, 4 hours was a good incubation time for all of the temperatures.

The 65 C temperature used in all other studies in this chapter served as a precaution. Other hydrolytic enzymes of barley are denatured at this temperature; alpha-amylase alone survives. However, there is information available, as mentioned previously, that indicates that cross-linked dye labelled substrates are truly impervious to beta-amylase. This information and the results of this study indicated that the BPA assay can be used at any temperature that fits the needs or the resources of the researcher.

Study VI: Alpha-Amylase Activity Measured by the BPA Assay vs. Germination Time

Alpha-amylase increases exponentially in germinating barley seeds, reaching a peak when the acrospire reaches 20 mm in length (Briggs, 1968). In this study, alpha-amylase activity, as measured by the BPA assay, increased linearly through 4 days of germination (acrospire 15 mm) (Figure 3-15). This linear relationship is due to alpha-amylase area not being directly proportional to alpha-amylase activity but rather the $\log_{10}$ of alpha-amylase activity.

Using the prediction equation, $\hat{Y} = -21.24 + 26.49x$ (from Figure 3-2), the alpha-amylase areas ($\hat{Y}$) in Figure 3-15 were converted to alpha-amylase activity (CBA dye release units, x) as described in Study IV of this chapter. When CBA dye release units were plotted vs. germination time in Figure 3-16, the exponential relationship previously reported (Briggs, 1968 and Groat and Briggs, 1969) was observed.

Study VII: Alpha-Amylase Activity Measured by the BPA Assay vs. Percent Sprouted Seed

The correlation of the alpha-amylase areas determined by the BPA assay with percent sprouted seed served as an additional method to determine the precision and accuracy of this assay. The relationship (Figure 3-17) between percent sprouted seed (1-6%) and alpha-

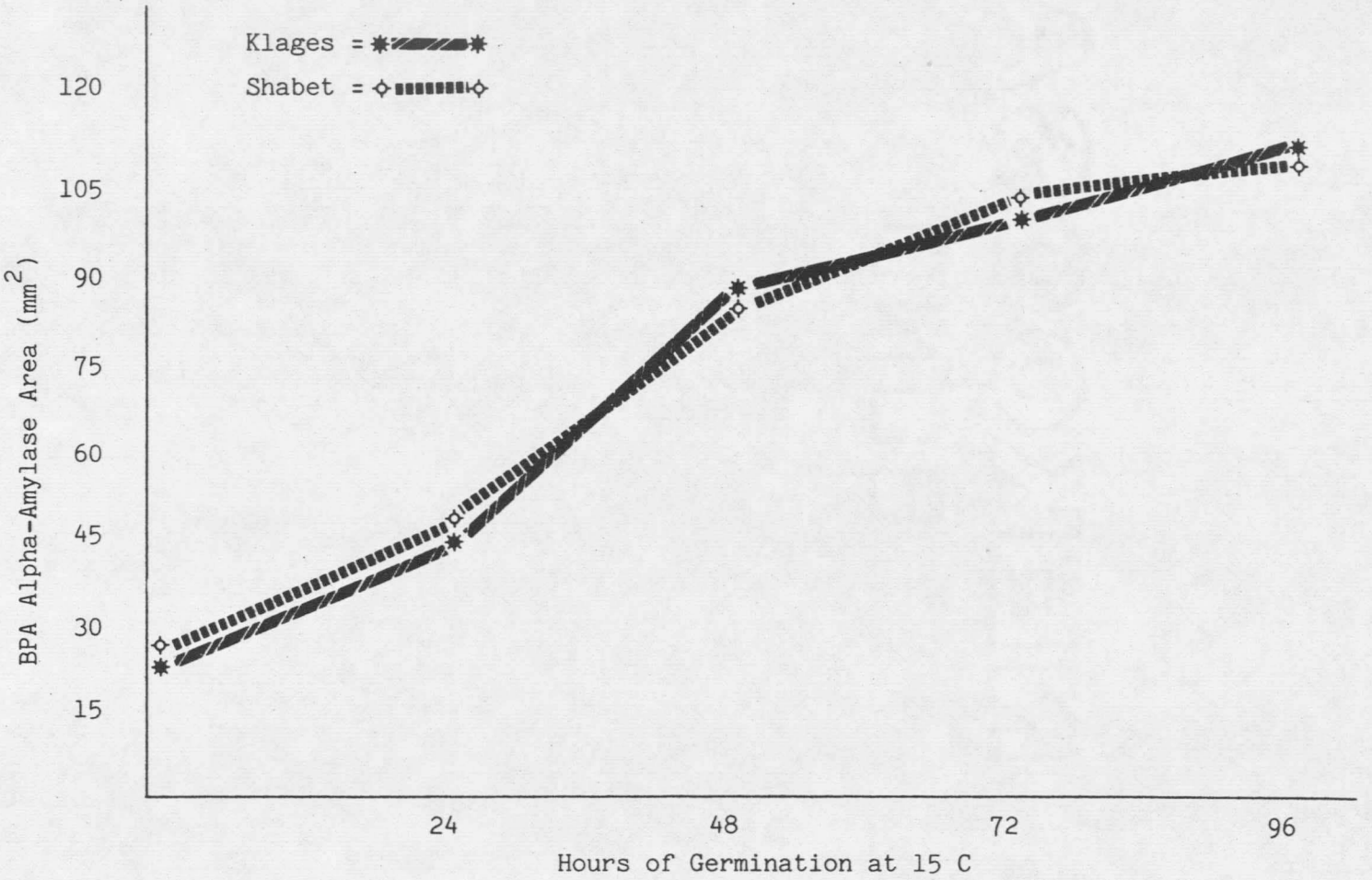

Figure 3-15. Comparison of BPA alpha-amylase area at 1 hour of incubation to alpha-amylase development during germination of two barley cultivars.

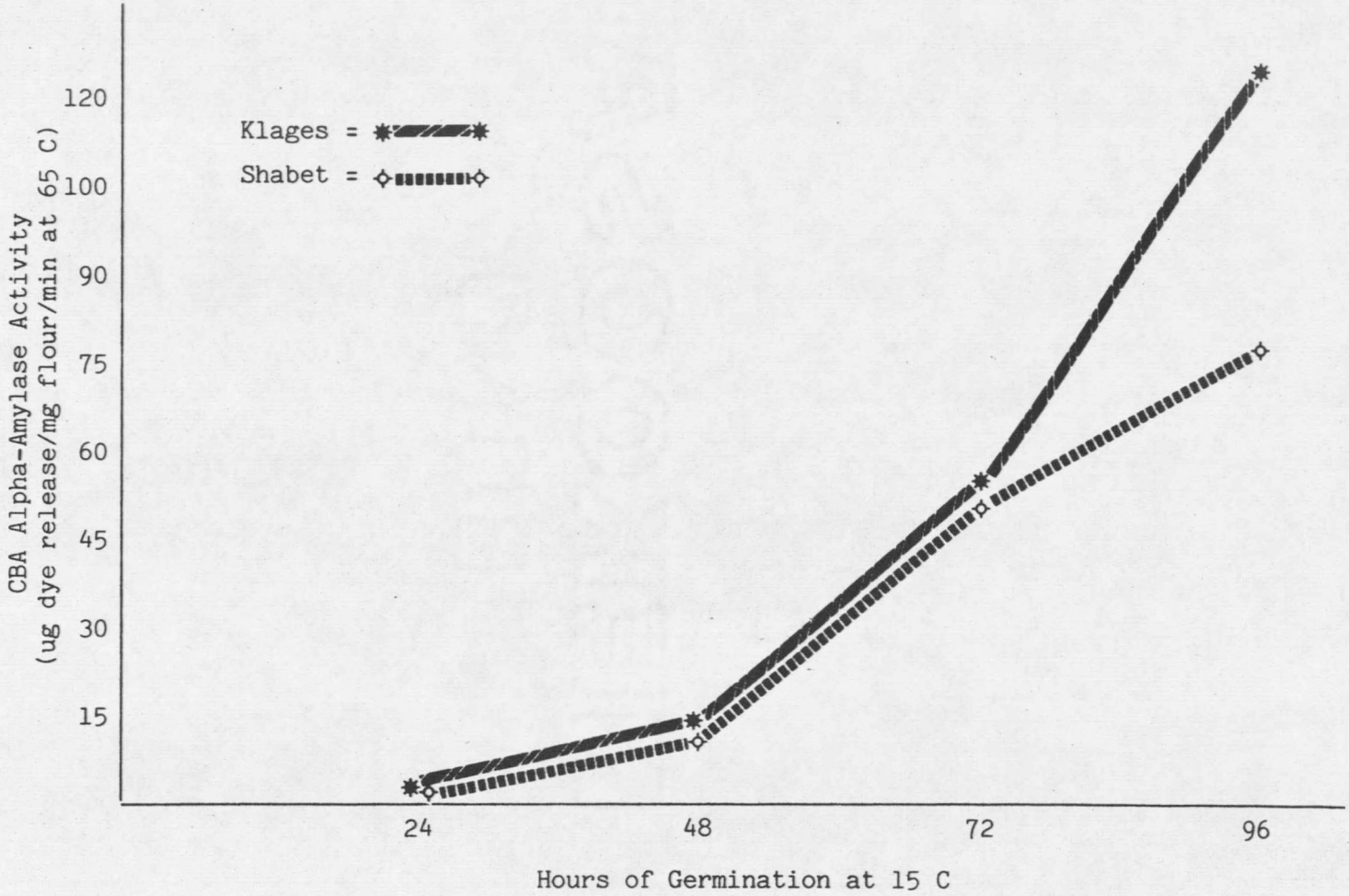

Figure 3-16. Comparison of CBA alpha-amylase activity as measured by the BPA assay to alpha-amylase development during germination of two barley cultivars.

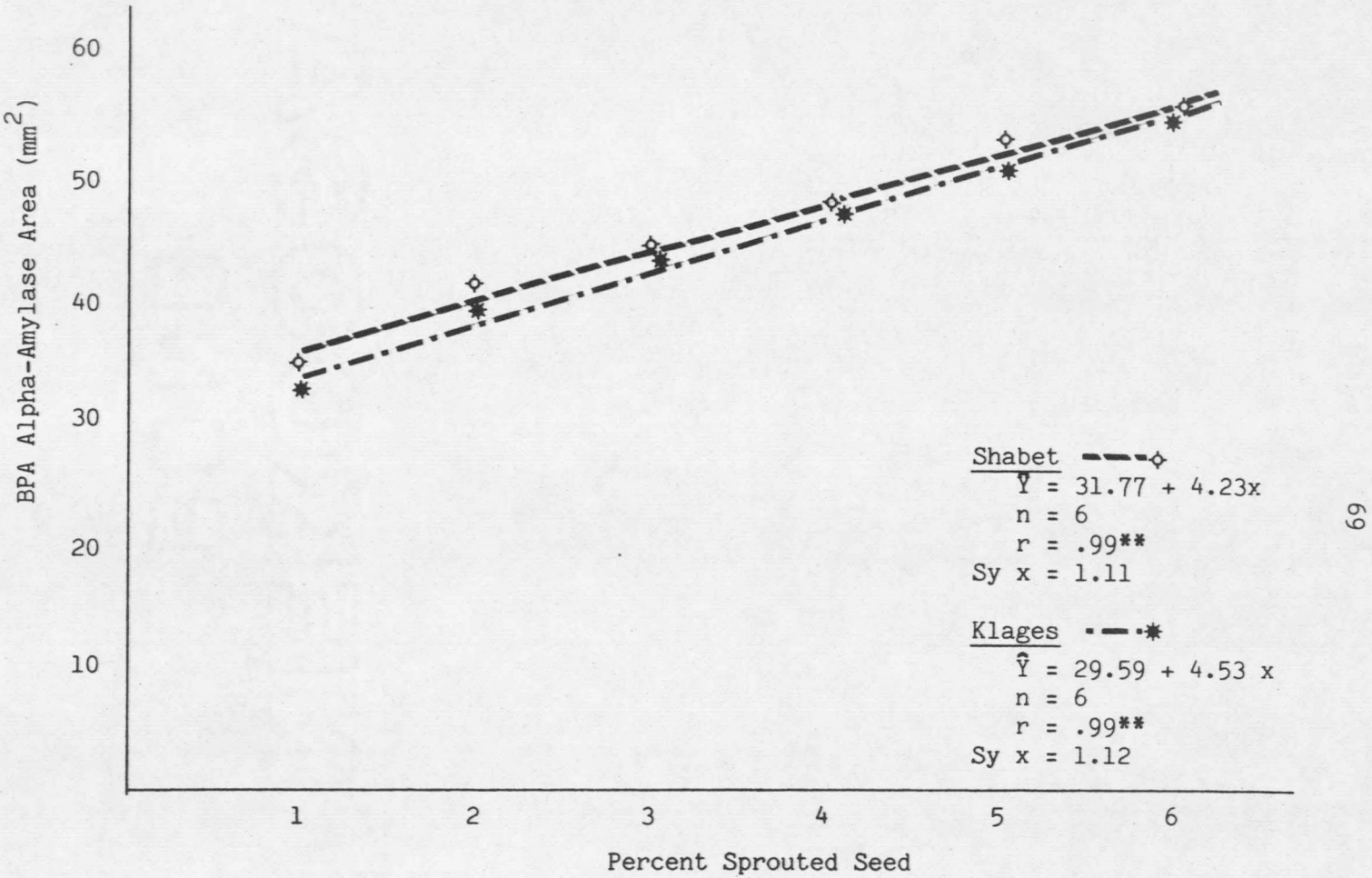

Figure 3-17. Regression of BPA alpha-amylase area at 1 hour of incubation to the percent sprouted seed (1-6%) in two barley cultivars.
**Significant at the 0.01 level.

amylase area is excellent for both Klages and Shabet barley ($r = .99^{**}$). The assay was most sensitive to the addition of just one sprouted seed. The increase in alpha-amylase area from 0-1% sprouted seed was sufficient to cause nonlinearity, and as a result, it was left out of the regression determination. This was due to alpha-amylase area not being directly proportional to alpha-amylase activity, but to the $\log_{10}$ of alpha-amylase activity. This means that the assay will be very sensitive to small changes in alpha-amylase at the lower levels of activity (0-1% sprouted seed) but increasingly less sensitive as enzyme activity increases. It is probable that by adding purified starch (without enzyme activity) to the barley flour samples being assayed, they would be brought into this more sensitive range. Further research on this is suggested.

Study VIII: The Use of the BPA Assay in Distinguishing Between the Malt Alpha-Amylase Activity of Feed and Malting Barley.

In Studies VI and VII of this chapter, Klages and Shabet were included only as two representative malting cultivars. No attempt was made to distinguish between the two. However, it is known that upon malting Klages will produce 40-50% more alpha-amylase than Shabet or Betzes (Hockett, personal communication). Thus, this presented another means to evaluate the BPA assay at levels of extremely high activity.

The cultivars, Compana and Titan, both feed barleys, had the lowest alpha-amylase activities (Figure 3-18). Betzes, a malting barley, was nonsignificantly higher than either of these two. This is not suprising since Betzes is not noted for high enzyme production. The difference between Klages and Betzes was exactly as expected. Klages, a malting barley recognized for high enzyme production, was significantly higher in activity than Betzes (50% higher). Thus, the BPA assay was effective for distinguishing between differences in enzyme activity at very high activity levels.

A rather large $LSD_{.05}$ was associated with this material. This was due to alpha-amylase area being related to the $\log_{10}$ of actual alpha-amylase activity. Thus, as activity increased, the BPA assay lost precision.

Originally this assay was developed to measure alpha-amylase activity in nongerminated seed. During the initial development of the assay, agar plates were made which had twice as much dyed starch, amylose-azure blue (enzyme substrate), than the present recipe calls for. These plates were insensitive to the low levels of activity in nongerminated seed, so the substrate was reduced. However, it is possible that this type of plate, with double substrate, would give more precision when measuring samples with high enzyme activity. Further research is suggested.

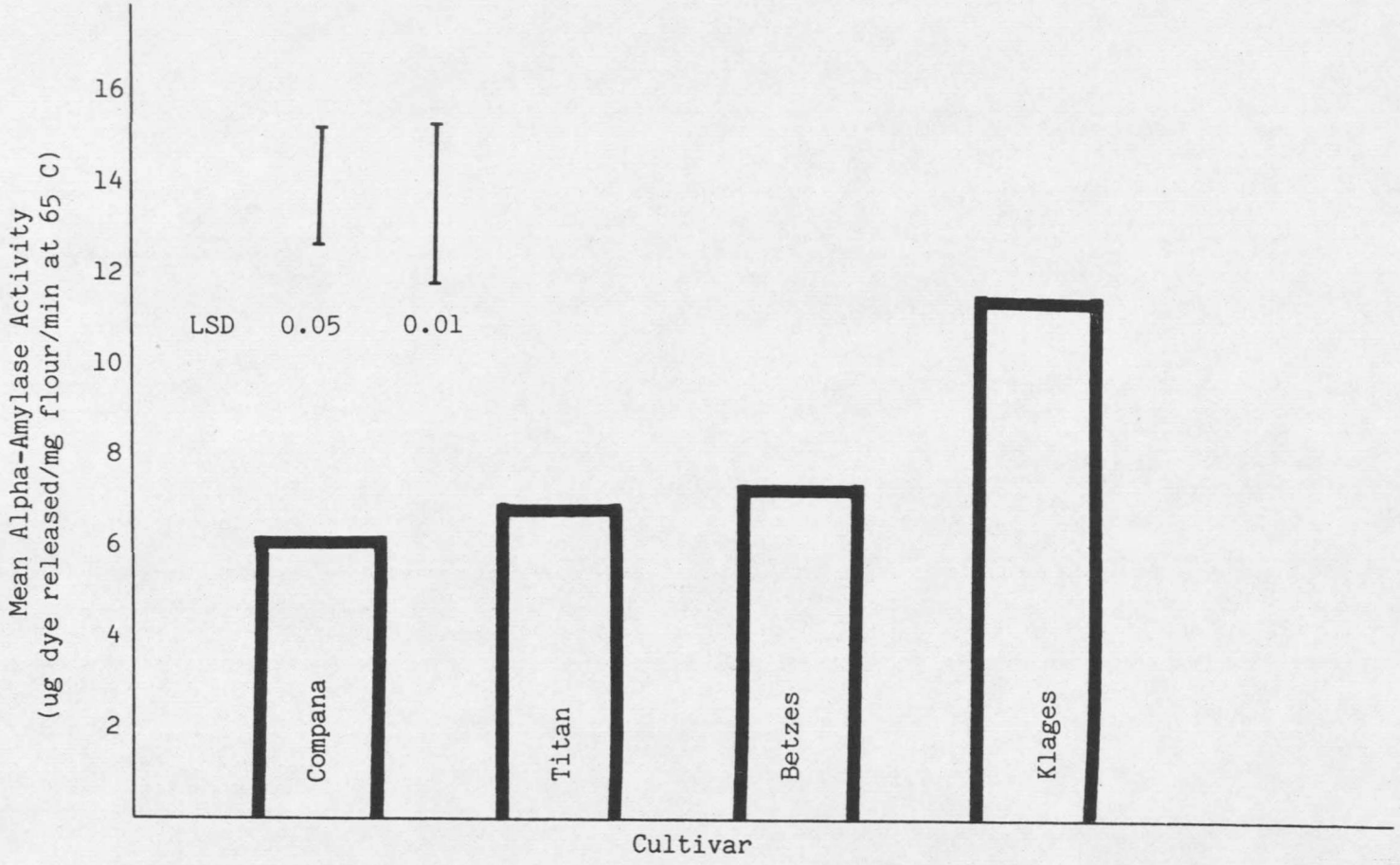

Figure 3-18. Mean alpha-amylase as measured by the BPA assay of malted barley samples of four cultivars grown in eight environments.

Summary

The BPA assay was highly correlated with standard assays for alpha-amylase 1) BPA assay vs. CBA assay: 4 hour incubation r = .99**; 2) BPA assay vs. IDC assay: 4 hour incubation r = .89**; and 3) BPA assay vs. Degress Lintner Reducing Power: 4 hour incubation r = .99**. The individual BPA plates were uniform in measurement of alpha-amylase activity (no significant plate-to-plate or within-plate variability on non-warped plates).

The BPA assay was not directly related to alpha-amylase activity but to the $\log_{10}$ of activity as determined by other assays (CBA, IDC, and Degrees Lintner Reducing Power). Thus, it was sometimes necessary to convert the BPA alpha-amylase areas to these units for direct comparisons between experiments. This was easily done through predication equations obtained by placing check samples on every plate.

The BPA assay could be used at any temperature between 25-65 C, and any time from 1-10 hours. The overall optimum temperature for all types of samples from high to low activity was 55 C. For samples with high activity, 65 C gave the maximum areas but 35 C gave the maximum differential. For samples of lowest activity, 55 C gave both maximum area and maximum differential among samples. As incubation time increased, so did the differential among samples. While 10 hours gave

the best differential among samples at all temperatures, 4 hours of incubation provided a compromise between accuracy and efficiency.

The BPA assay accurately followed alpha-amylase accumulation in germinating Shabet and Klages barley. Percent sprouted seed in samples of Klages and Shabet barley were accurately predicted by this assay (BPA assay vs. percent sprouted seed, $r = .99^{**}$). The BPA assay also distinguished high alpha-amylase malt (Klages) from low alpha-amylase malt (Betzes).

CHAPTER 4

THE CHARACTERISTICS OF ALPHA-AMYLASE IN NONGERMINATED GRAIN SAMPLES OF WASHONUPANA AND OTHER BARLEYS

Introduction

Washonupana barley starch has been studied by Goering and Eslick (1976) and found to contain sufficient alpha-amylase activity to completely liquify itself in a Brabender Amylograph. This unique property is thought to be due to 1) unusually high levels of alpha-amylase in the seed, or 2) a different kind of alpha-amylase which readily attaches to starch granules.

The objectives of the research presented in this section were to 1) determine if ungerminated Washonupana barley contained unusually high levels of alpha-amylase, 2) determine if Washonupana has a unique isozyme of alpha-amylase, 3) determine the effect of waxy starch on alpha-amylase activity and starch digestibility, and 4) determine the extent to which alpha-amylase occurs in sound ungerminated barley across a range of genotypes.

Materials and Methods

Study I: Alpha-Amylase Activity in Washonupana vs. Compana

Eight paired grain samples of Washonupana and Compana and an additional three grain samples of Washonupana were grown in a wide range of environments.

Alpha-amylase activity of the eight paired samples was measured by the BPA assay described in Chapter 3, after 4 hours of incubation. The alpha-amylase areas were converted to CBA dye release units. The IDC method (MacGregor et al., 1971a, see acknowledgements) was used to obtain a second determination of alpha-amylase activity for the eight paired samples and the only determination of alpha-amylase activity for the three additional samples of Washonupana. The cultivar 'Conquest' (C.I. 11638) was used as a check for this latter method.

A third method of measuring alpha-amylase activity was performed on the eight paired samples using the reducing power of a starch/water digest (DeHaas et al., 1978). Ground barley (0.5g) was mixed in 10 ml of an acetate buffer, pH 5.4, containing 12 mm calcium acetate. The mixture was heated for 20 minutes at 70 C to destroy all enzyme activity except alpha-amylase. The samples remained in a 50 C water bath for 24 hours, and were then centrifuged. The supernatant was

saved and assayed for reducing substances (the result of alpha-amylase activity) by the 3,5-dinitrosalicylic acid method (Whelan, 1964).

The treatment means of the BPA assay and the reducing power assay were compared by a paired t-test.

Study II: Isozyme Analysis for Alpha- and Beta-Amylase Based Upon Isoelectric Focusing of Enzymes Derived From Washonupana Barley Grown at 11 Locations

The 11 samples of Washonupana used in Study I were used in this study. Isoelectric focusing was used to separate the isozymes of alpha- and beta-amylase in Washonupana barley (MacGregor, 1976, see acknowledgements). Isoelectric focusing is a method of precisely separating isozymes on the basis of electrophoresis across a pH gradient. An enzyme will migrate toward the positive or negative pole until it reaches the pH value at which it no longer carries a charge. The cultivar Conquest was used as a check for this method.

Study III: Comparison of Washonupana Barley with Other Waxy, Short-Awn, Hulless Isotypes of Compana Using the Isozyme Analysis for Alpha- and Beta-Amylase

Alpha- and beta-amylase isozymes were compared in Compana and five derived isotypes: Nupana, Shopana, Wapana, Wanupana, and Washonupana. The double isogenic, 'Washopana', was not available.

These genotypes were grown in unreplicated plots in different environments.

Alpha-amylase activity was measured on all samples using the IDC method (MacGregor et al., 1971a, see acknowledgements). The cultivar, Conquest, was included as a check for this assay. Isozyme analysis using the isoelectric focusing technique (MacGregor, 1976, see acknowledgements) was performed on all samples.

Study IV: Alpha-Amylase Activity vs. Starch Digestibility in Waxy Isotypes

Twenty paired grain samples of waxy and nonwaxy Compana, two paired grain samples of waxy and nonwaxy Titan, three paired grain samples of waxy and nonwaxy Betzes, and one paired grain sample of waxy and nonwaxy Oderbrucker were used to examine the effect of the waxy gene upon alpha-amylase activity and starch digestibility. The paired samples were unreplicated and grown in many environments.

The alpha-amylase activity of 16 paired samples of Wapana and Compana was measured using the BPA assay described in Chapter 3. Alpha-amylase areas were converted to CBA dye release units.

The alpha-amylase activity of six of the paired samples used in the BPA assay and of the rest of the samples described above was assayed using the flour digest reducing power assay (DeHass et al., 1978) described in Study I of this chapter.

All sample means were compared by a paired t-test.

Study V: Alpha-Amylase Activity of 50 Barley Cultivars

Twenty-five two-row and twenty-five six-row cultivars adapted to the western United States were grown in nonreplicated, four row plots in the environments found in Moccasin, Huntley, and Bozeman, MT in 1977. Both malting and feed types were included. None of the samples showed any sign of sprout damage.

A minor modification of the BPA assay was used to measure alpha-amylase activity. Small petri dishes were used instead of the large petri dishes, providing only 10 randomly placed samples per plate. Each sample was replicated twice with the replications being blocked in time, i.e., the second replication was run 3 weeks after the first. Sample diameters were measured at 2 hours and converted to alpha-amylase area. Significance of factors such as genotype, environment, and their interaction were determined by F-tests. Genotypes were considered as fixed and environments as random. Three orthogonal single degree-of-freedom comparisons were made to determine the effect of row type (six vs. two), and use type (malt vs. feed) within row types, upon alpha-amylase activity of these 50 genotypes. Comparisons were made using the classifications of row and use type in Table 4-9. Cultivars classified as both feed and malt types,

were classified for the comparisons according to their primary usage (not in parenthesis).

Results and Discussion

Study I: Alpha-Amylase Activity in Washonupana vs. Compana

Alpha-amylase activity, as measured by the BPA assay, was significantly higher for Washonupana than for Compana (Table 4-1). The activity in both cultivars was trivial when compared to malt (1/1,000 to 1/10,000 of malt). The difference was small enough that Washonupana being a smaller seeded, hulless barley could account for the difference. Alpha-amylase would be located primarily in the aleurone of nongerminated seed and a pound of Washonupana would have more aleurone than a pound of Compana, due to the reduced seed size. Therefore, it would have more alpha-amylase.

Washonupana alone was also assayed by the IDC method for alpha-amylase activity (Table 4-2). With the exception of sample 9 (massive sprout damage) and sample 10 (slight sprout damage) no sample of Washonupana showed exceptional activity. Most samples were similar to Conquest, a six-row malting barley. Any IDC values below 100 represent trivial amounts of alpha-amylase well within the range of activity found in normal sound barley (MacGregor, personal communication).

Table 4-1. BPA alpha-amylase activity of eight Compana-Washonupana pairs.

Pair No.	Alpha-Amylase Activity (ng dye released/mg flour/min at 65 C)	
	Compana ($\bar{x}_1$)	Washonupana ($\bar{x}_2$)
1	18.28	19.52
2	19.52	29.51
3	22.25	19.52
4	19.52	27.52
5	18.36	27.41
6	23.84	32.20
7	18.28	34.20
8	15.82	28.84
$\bar{x}$	19.48	26.34
$\bar{x}_1 - \bar{x}_2$	-6.86*	

*Significant at the 0.05 level.

Table 4-2. IDC alpha-amylase activity of 11 Washonupana samples and a Conquest check.

Sample No.	Condition of Sample	Alpha-Amylase Activity (IDC units/g)
1	Sound Barley	20
2	Sound Barley	22
3	Sound Barley	50
4	Sound Barley	17
5	Sound Barley	8
6	Sound Barley	20
7	Sound Barley	62
8	Sound Barley	26
9	Massive Sprout Damage	62,640
10	Received Moisture in Swathed Rows	131
11	Sound Barley	0
Check	Conquest	46

The IDC and BPA assays indicated that Washonupana was not producing exceptional amounts of alpha-amylase on an absolute basis. The only really significant increases arose through the ordinary environmental factors that affect all barleys, i.e., moisture at harvest and associated sprouting.

The reducing power assay (Table 4-3), however, indicated a much larger difference in activity between Washonupana and Compana. Washonupana had 2.5 times more activity, as measured by this assay, than Compana. This difference may not be due to alpha-amylase activity, but rather, to an interaction between the endogenous alpha-amylase and starch. Since waxy starch is more digestible than normal starch, upon digestion the waxy isotypes should produce more reducing ends than nonwaxy types if both have similar amounts of enzyme.

The BPA and IDC assays measure the effect of alpha-amylase derived from grain upon an external substrate. The reducing power assay measures endogenous enzymes acting on endogenous starch. The effect of substrate cannot be separated from the effect of enzyme with this assay. As a measure of enzyme activity, the reducing power assay should be used with caution.

Table 4-3. Reducing power alpha-amylase activity of eight Compana-Washonupana pairs.

Pair No.	Alpha-Amylase Activity (mg maltose/ 0.5 g flour/24 hours at 50 C)	
	Compana ($\bar{x}_1$)	Washonupana ($\bar{x}_2$)
1	4	8
2	3	9
3	2	7
4	3	4
5	3	6
6	2	6
7	3	6
8	2	7
x	2.75	6.63
$\bar{x}_1 - \bar{x}_2$	-3.88**	

**Significant at the 0.01 level.

Study II: Isozyme Analysis for Alpha- and Beta-Amylase Based Upon Isoelectric Focusing of Enzymes Derived from Washonupana Barley Grown at 11 Locations

There is essentially no difference in banding patterns for combined alpha- and beta-amylase among any of the samples of Washonupana and the Conquest check (Figure 4-1). The sprout damaged sample from the environment at Kalispell, MT had intensive activity over the entire range of isozymes.

The isozyme profile for alpha-amylase alone is shown in Figure 4-2. The isozymes are broken into three major bands. Alpha-amylase I is the predominant form found in the developing seed, often called green alpha-amylase. Alpha-amylase II and III are formed upon germination. There are no consistent differences between the profile observed for Conquest and the 11 Washonupana samples.

The massively sprout damaged Kalispell and Conrad, MT samples had elevated levels of all isozymes, especially II and III. Bruckner and Eslick (1979) have reported that Washonupana is especially susceptible to post-harvest sprouting due to its lack of post-harvest dormancy.

Study III: Comparison of Washonupana Barley With Other Waxy, Short-Awn, Hulless Isotypes of Compana Using the Isozyme Analysis for Alpha- and Beta-Amylase

Washonupana was not uniquely endowed with unusual amounts or special types of alpha-amylase. The data presented in Table 4-4

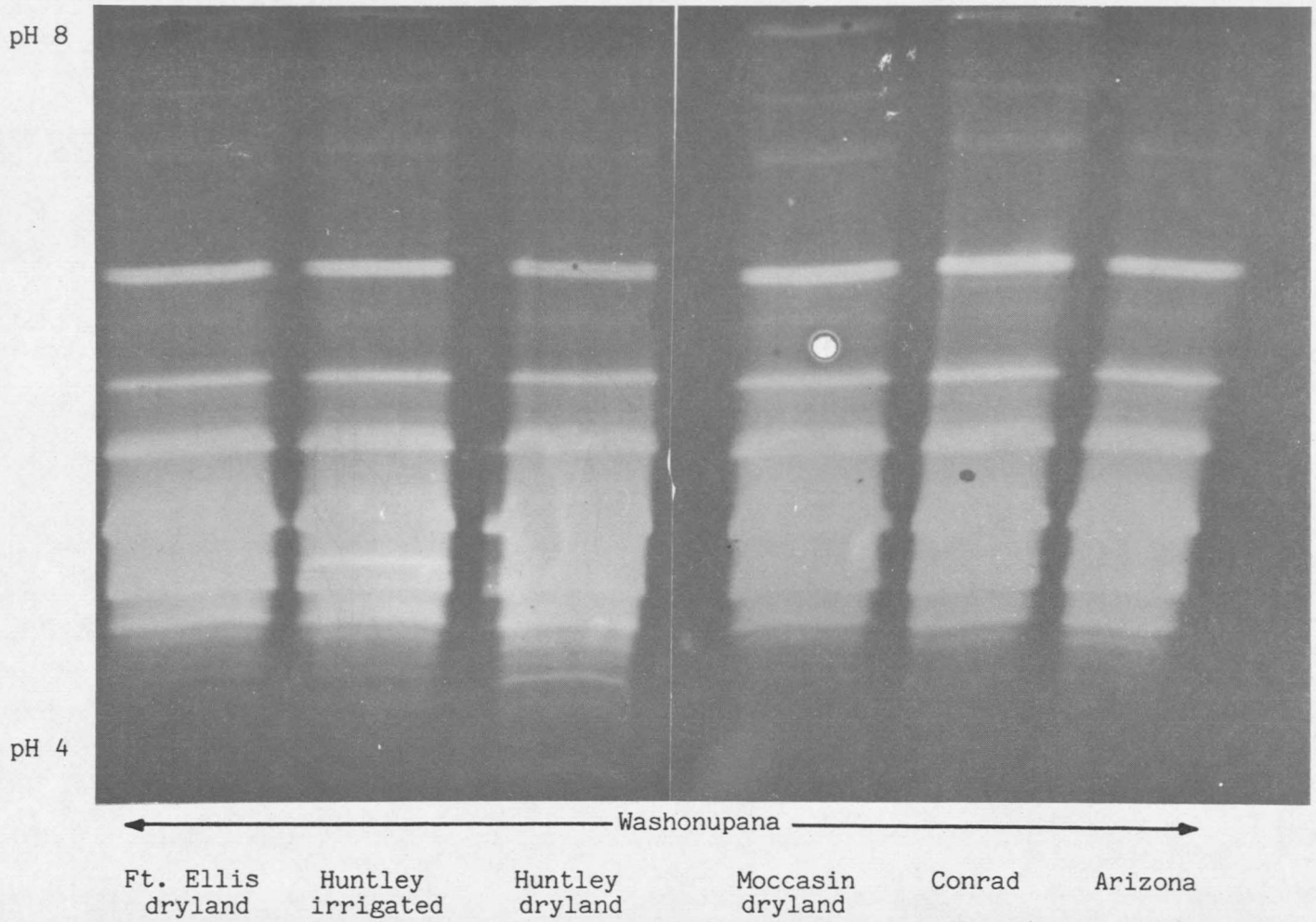

Figure 4-1. Alpha- and beta-amylase zymogram of Washonupana grown in 11 environments and a Conquest check after isoelectric focusing on a pH 4-8 gradient.

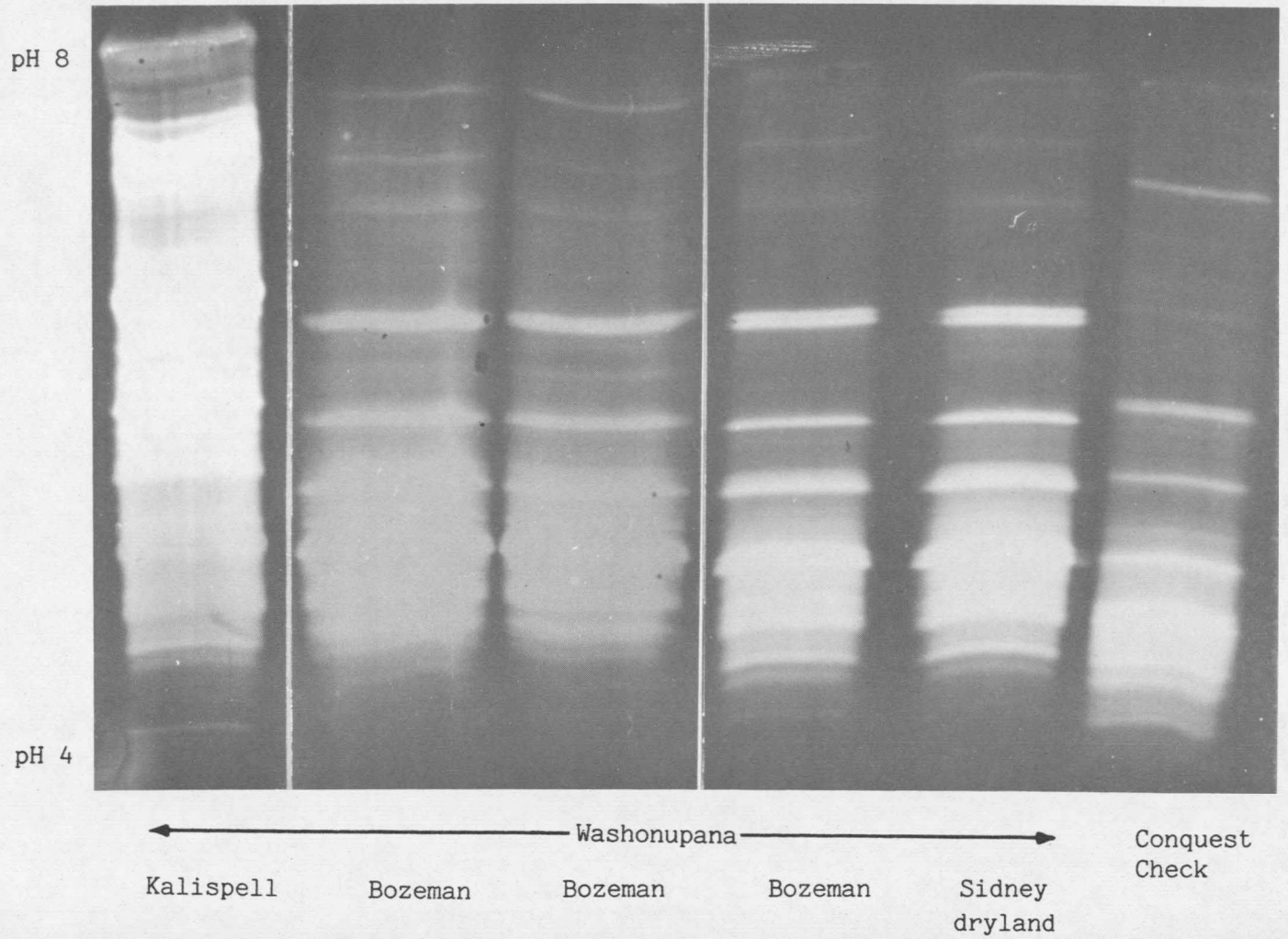

Figure 4-1 (Continued)

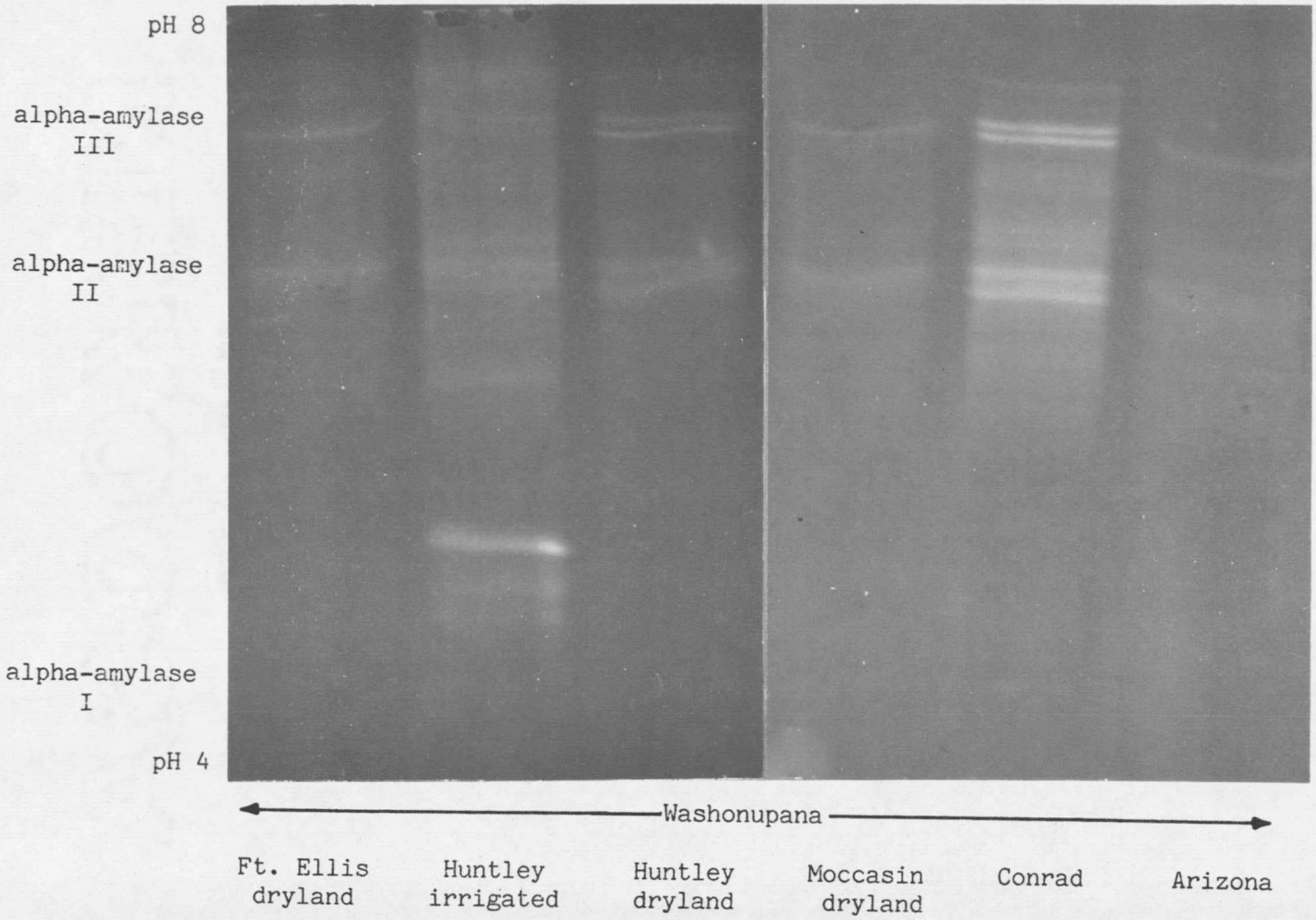

Figure 4-2. Alpha-amylase zymogram of Washonupana grown in 11 environments and a Conquest check after isoelectric focusing on a pH 4-8 gradient.

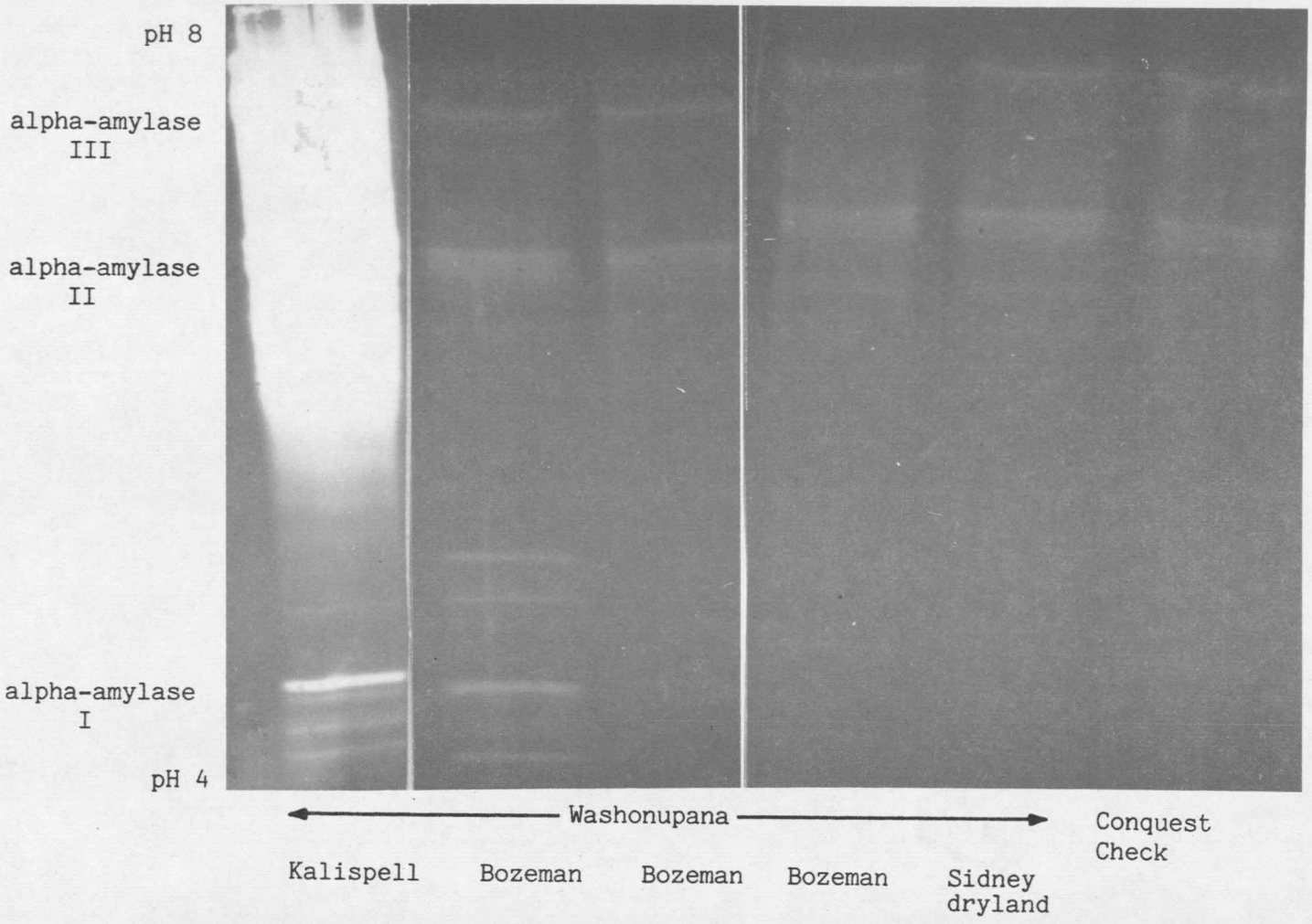

Figure 4-2 (Continued)

Table 4-4. IDC alpha-amylase activity of six component isotypes of Washonupana and a Conquest Check.

Isotypes	Alpha-Amylase Activity (IDC units/g)
Compana	6
Wapana	0
Shopana	55
Nupana	12
Shonupana	53
Wanupana	19
Conquest	46

indicates that none of the component isotypes of Washonupana were high in alpha-amylase. Most have lower activity than the Conquest check. No unique isozymes or amounts of alpha- and beta-amylase were observed (Figure 4-3). The weak alpha-amylase bands in Figure 4-4 reflect the low amounts of enzyme detected by the IDC test. None of the isotypes had an isozyme pattern different from Washonupana. Most of the activity present was found in alpha-amylase II and III.

Study IV: Alpha-Amylase Activity vs. Starch Digestibility in Waxy Isotypes

It was suggested in Study I that the difference between Washonupana and Compana using the reducing power assay was due to an interaction between waxy starch and small amounts of alpha-amylase. If this is the case, it should be observed in other waxy isotypes of Compana and in waxy isotypes of other cultivars.

The BPA assay alpha-amylase activity (Table 4-5) indicated low levels of activity and no significant differences were found between Wapana and Compana. Wapana, like Compana, is a hulled, long-awn cultivar, and thus, has a similar seed size to Compana. It is not surprising that the small but significant difference between Washonupana and Compana is not observed here.

Wapana produced three times more reducing power than Compana, after a self-digest (Table 4-6). The same general pattern was ob-

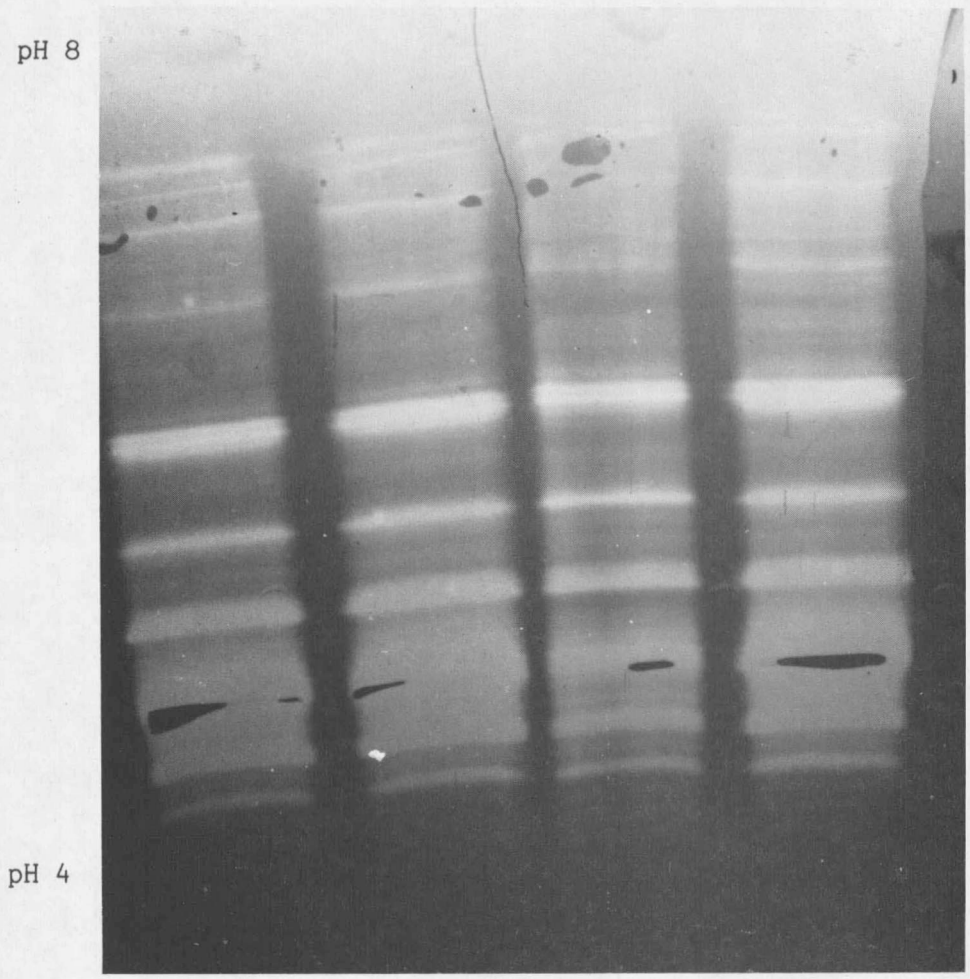

Figure 4-3. Alpha- and beta-amylase zymogram of all available waxy, short-awn, hulless isogenics of Compana after isoelectric focusing on a pH 4-8 gradient.

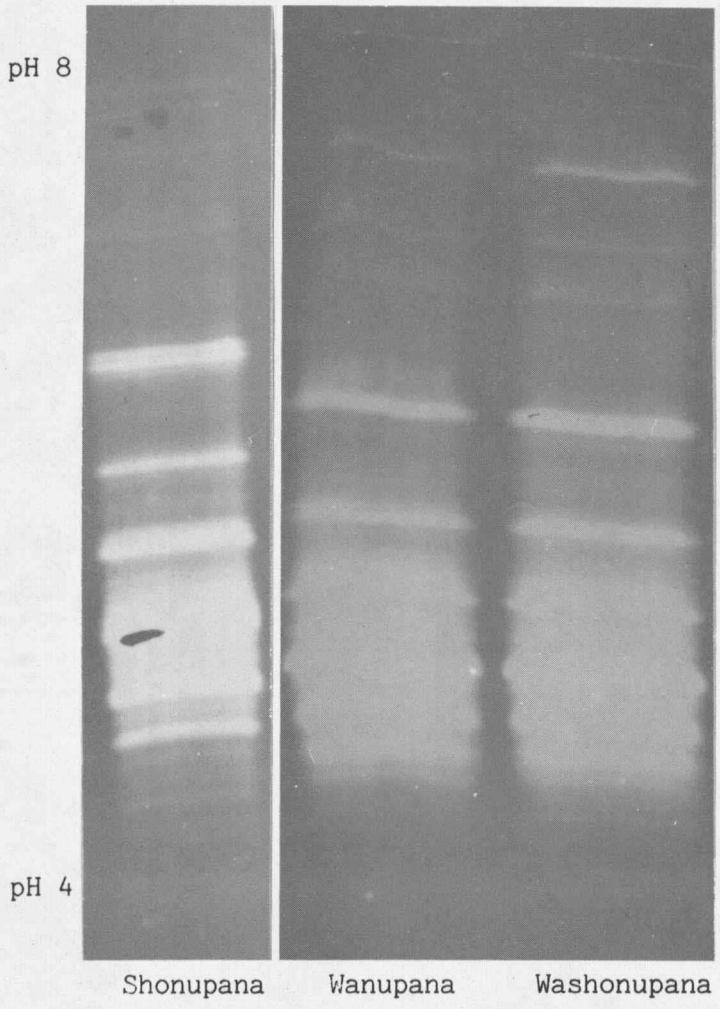

Figure 4-3 (Continued)

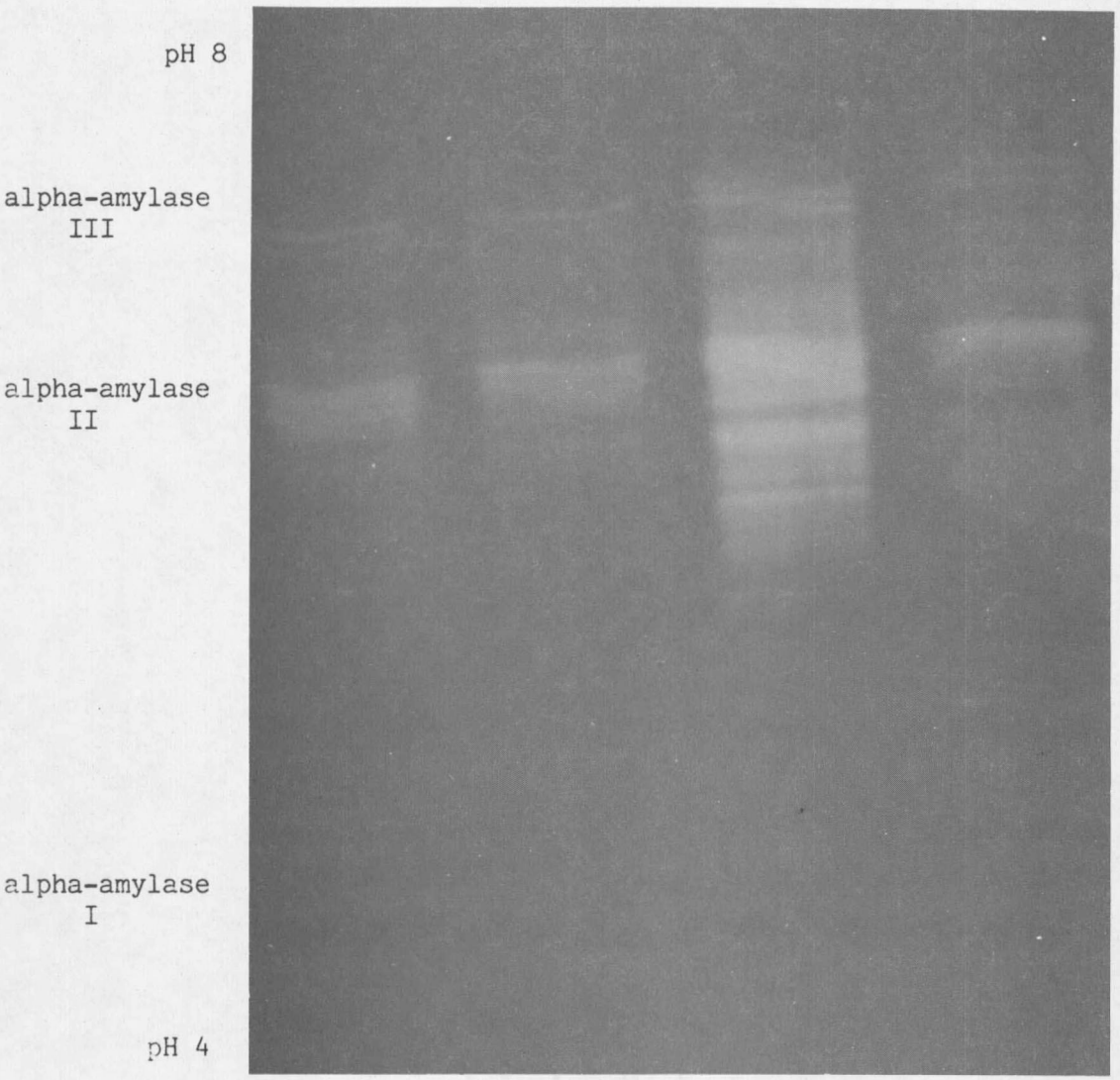

Figure 4-4. Alpha-amylase zymogram of all available waxy, short-awn, hulless isogenics of Compana after isoelectric focusing on a pH 4-8 gradient.

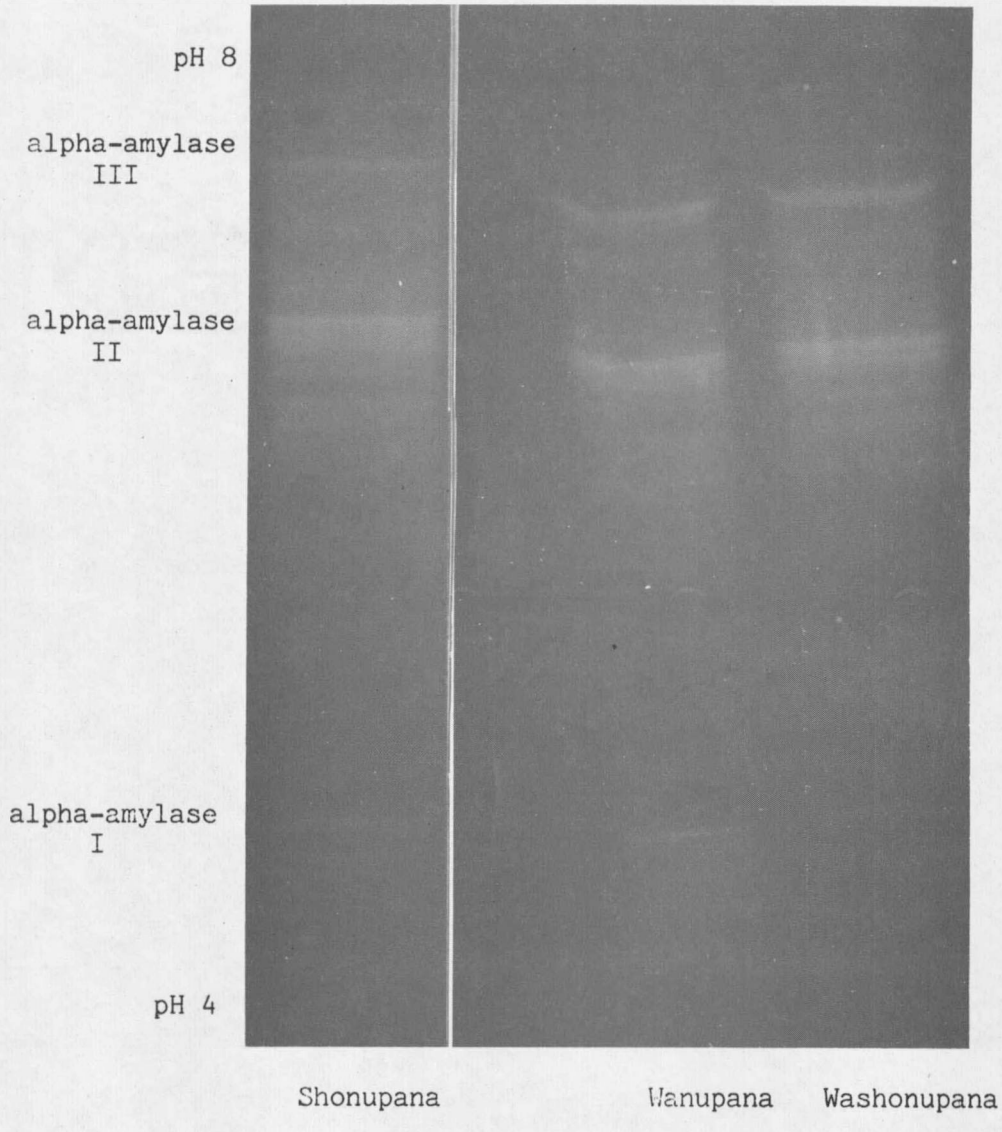

Figure 4-4 (Continued)

Table 4-5. BPA alpha-amylase activity of 16 waxy-nonwaxy Compana pairs.

Pair No.	Alpha-Amylase Activity (ng dye released/mg flour/min at 65 C)	
	nonwaxy ($\bar{x}_1$)	waxy ($\bar{x}_2$)
1	29.02	36.27
2	29.02	29.02
3	29.02	23.56
4	36.27	36.27
5	23.56	29.02
6	36.27	45.96
11	23.56	19.40
12	29.02	29.02
13	29.02	29.02
14	23.56	29.02
15	23.56	23.56
16	23.56	29.02
17	19.40	15.22
18	15.22	23.56
19	29.02	36.27
20	36.27	36.27
$\bar{x}$	27.209	29.404
$\bar{x}_1 - \bar{x}_2$	-2.20 N.S.	

Table 4-6. Reducing power alpha-amylase activity of 10 waxy-nonwaxy Compana pairs.

	Alpha-Amylase Activity (mg maltose/ 0.5 g flour/24 hours at 50 C)	
Pair No.	nonwaxy ($\bar{x}_1$)	waxy ($\bar{x}_2$)
1	2	6
2	1	3
3	1	2
4	2	5
5	1	4
6	2	8
7	4	11
8	2	7
9	3	8
10	3	9
$\bar{x}$	2.1	6.3
$\bar{x}_1 - \bar{x}_2$	-4.2**	

**Significant at the 0.01 level.

served with waxy isotypes in three other cultivars (Table 4-7). They produced about twice as much reducing power as the normals.

Goering et al. (1973a) observed that when waxy starch is extracted from barley by wet milling, cracks and fissures occur in the waxy starch while the normal starch remains intact. Light microscope examination was made of waxy and normal starch, isolated from sound seed of Titan, Compana, Betzes, Oderbrucker, and 'Hector' genotypes. In all cases the waxy types not only exhibited the fissures described by Goering et al. (1973a), but numerous pits were also observed. The pits were identical in appearance to the digestion pin holes that occur when starch granules are degraded by alpha-amylase. The non-waxy counterparts, in all cases, remained completely intact with no evidence of starch granule degradation.

MacGregor and Ballance (1980) suggested that the self-liquefying phenomenon of Washonupana barley was due to the extreme ease with which waxy barley can be degraded. In the wet milling process, the small amounts of enzyme in Washonupana barley may begin to degrade the highly digestible waxy starch granules, and once the granules are penetrated, the alpha-amylase failed to be washed out.

Although Washonupana barley contains only small amounts of alpha-amylase at harvest, on a weight basis, it has significantly more

Table 4-7. Reducing power alpha-amylase activity of six waxy-nonwaxy pairs within three genotypes.

Pair No.	Cultivar	Alpha-Amylase Activity (mg maltose/0.5 g flour/24 hours at 50C) Nonwaxy ($\bar{x}_1$)	Waxy ($\bar{x}_2$)
1	Oderbrucker	5	12
2	Titan	7	10
3	Titan	6	12
4	Betzes	4	8
5	Betzes	3	5
6	Betzes	2	4
$\bar{x}$		4.5	8.5
$\bar{x}_1 - \bar{x}_2$		-4.0**	

**Significant at the 0.01 level.

activity than Compana. This small but significant increase in activity, in combination with the more digestible starch, may explain why some samples of Washonupana tend to possess self-liquefying properties when examined in a Brabender Amylograph.

Study V: Alpha-Amylase Activity of 50 Barley Cultivars

Highly significant differences were detected among the 50 cultivars and among the three environments for alpha-amylase activity (Table 4-8). The environment x genotype interaction was nonsignificant. Highly significant differences in alpha-amylase area were detected between two-row (mean = 18.40 mm^2) and six-row (mean = 22.27 mm^2) cultivars, and within the six-row cultivars, between malt (mean = 25.69 mm^2) and feed (mean = 20.73 mm^2) types. Within the two-row cultivars, no difference was detected between the malt (mean = 17.43 mm^2) and feed (mean = 18.88 mm^2) types.

A list of the 50 cultivars in descending order of alpha-amylase activity, measured as alpha-amylase area, is shown in Table 4-9. The 11 cultivars with the highest alpha-amylase activity were all six-row barleys. However, the lowest ranked cultivar was 'Trebi', also a six-row barley. The third highest ranking two-row barley was Klages, the western two-row malting barley known for high enzyme production. Prior to the development of Klages, two-row cultivars were not noted

Table 4-8. Analysis of variance and coefficient of variation (C.V.) for BPA alpha-amylase area (mm^2/2 hours at 65 C) of 50 cultivars grown in three environments.

Source of Variance	df	SS	MS	F
Replication	1	1122.00	1122.00	35.25**
Environment (E)	2	257.50	128.80	4.05**
Genotype (G)	49	4756.70	97.08	3.25**
Two-row vs. six-row	1	1061.67	1061.67	35.57**
Six-row malt vs. feed	1	742.95	742.95	24.89**
Two-row malt vs. feed	1	73.19	73.19	2.45
G x E	98	2924.90	29.85	0.94
Error	149	4743.70	31.83	
C.V. % = 27.7				

**Significant at the 0.01 level.

Table 4.9. Mean BPA alpha-amylase area and commercial use (malt = M; feed = F) of 25 six-row and 25 two-row barley cultivars.

Cultivar	C.I. Number	Row Type	Use Type	Alpha-Amylase Activity (mm^2/2 hours at 65 C)
Larker	10648	6	M	30.66
Trophy	10647	6	M	29.86
Harlan	7008	6	F	29.79
Dickson	10968	6	M	25.54
Titan	7055	6	F(M)	25.45
Montcalm	7149	6	M	24.77
Beecher	6566	6	F	24.58
Nordic	15216	6	M	24.54
Primus II	13796	6	M	23.77
Galt	11770	6	F(M)	23.71
Hiland	9530	6	F	23.61
Vireo	15555	2	F	22.61
Horn	926	2	F	22.58
Klages	15478	2	M	21.75
Glacier	6976	6	F	21.68
California Mariout	1455	6	F	21.68
Firlbecks III	10088	2	F	20.75
Hector	15514	2	F(M)	20.75
Herta	8097	2	F	20.75
Unitan	10421	6	F	20.75
Traill	9538	6	M	20.72
Atlas	4118	6	F	20.72
Steptoe	15229	6	F	20.72
Erbet	13826	2	M	20.72
Briggs	13682	6	F	19.85
Dekap	3351	2	F	19.82
Gem	7243	6	F	19.82
Ingrid	10083	2	F	19.78
Frontier	7155	6	F	19.78
Marie	11334	2	F	19.65
Compana	5438	2	F	18.85
Otis	7557	2	F	18.85
Betzes	6398	2	M	18.02
New Moravian	10107	2	M	18.02
Bonneville	7248	6	F	18.02

Table 4-9 (Continued)

Cultivar	C.I. Number	Row Type	Use Type	Alpha-Amylase Activity (mm^2/2 hours at 65 C)
Liberty	9549	6	F	17.95
Zephyr	13667	2	F	17.38
Vantage	7324	6	F	17.18
Freja	7130	2	F	17.12
Hannchen	531	2	M	17.08
Maris Mink	15556	2	F	17.08
Georgie	-	2	F	16.38
Piroline	9558	2	M	16.18
Heines Hanna	9532	2	M	16.16
Haisa II	10420	2	M	16.15
Munsing	6009	2	F	15.32
Steveland	13100	6	F	15.28
Spartan	5027	2	F	14.42
Vanguard	11868	2	M	12.78
Trebi	936	6	F	12.71

$L.S.D._{0.01} = 8.52$; $L.S.D._{0.05} = 6.45$.

for high enzyme production. But, these results indicate that 'Horn' and 'Vireo' could serve as parental sources of high enzyme activity within a two-row background.

It is evident from these results that the presence of alpha-amylase in sound, ripe barley is not unique to Washonupana, but is common to barley in general. Differences between genotypes appeared to be related to usage, especially within six-row cultivars. The six-row malt cultivars, noted for high enzyme production when malted, had higher amounts of alpha-amylase activity in nongerminated seed than the feed types. Further research is suggested to determine that this relationship holds over genotypes and environments.

Summary

Washonupana does not have abnormally high levels of alpha-amylase at harvest. It does, however, contain a significantly higher level of this enzyme than Compana, on a weight basis. The actual amount of alpha-amylase present at harvest was about 1/1,000 to 1/10,000 of that found in malted grain.

The waxy starch found in Washonupana and the waxy isotypes of Compana, Titan, Betzes, and Oderbrucker were significantly more susceptible than normal starch to digestion by alpha-amylase present in ripe seed by a factor of 2-3:1.

Unique alpha- or beta-amylase isozymes were not detected in Washonupana barley or in any isotypes involving the waxy, short-awn, or hulless genes (singly or in combination).

Small amounts of alpha-amylase were detected in all samples of 50 barley cultivars grown at three environments. Significant differences among cultivars and environments were observed. It was possible to distinguish malting from feed cultivars using the BPA assay, indicating it would be a useful test in a malting barley breeding program.

The self-liquefying property of Washonupana appeared to result from the action of small amounts of alpha-amylase, that occur to varying amounts in barley at harvest, upon the very digestible waxy starch. This suggests that with the waxy starch and the higher levels of alpha-amylase found in malting cultivars, it would be possible to produce other cultivars with the self-liquefying property of Washonupana.

CHAPTER 5

THE EFFECT OF WAXY ENDOSPERM UPON MALTOSE PRODUCTION IN MALTED BARLEY

Introduction

Of the two molecular species that make up starch, amylopectin is more digestible by heat, enzyme, or acid than amylose (Goering et al., 1981). It is hydrolized 20 times more rapidly than amylose when digested in vitro by enzyme (Harris, 1962a and Goering et al., 1981). In malting or germination the amylopectin portion of the starch granule is preferentially hydrolyzed over the amylose (Baker and Hulton, 1938). In fact, little or no amylose is hydrolized during the malting process (Hall et al., 1956). Since waxy barley is essentially 100% amylopectin, upon malting, it should result in a more highly modified malt. This should present certain advantages to the maltster and brewer.

The purpose of this reserach was to determine 1) the effect of the waxy vs. nonwaxy isotypes on production of maltose measured at various stages of the malting process, and 2) the influence that background genotype has on the expression of the waxy trait with regard to starch digestibility.

Materials and Methods

General

The barley cultivars used in the following studies were covered, waxy and nonwaxy pairs of Betzes, Titan, and Compana. The derivation and naming of the waxy isotypes was explained in Chapter 2. The variety Klages was included in Study III since it is the current industrial standard for two-row malting barley.

Study I: The Influence of Germination Interval Upon Maltose Production in Waxy vs. Nonwaxy Compana

Waxy and nonwaxy Compana were grown in paired plots in 15 Montana environments with no field replication. Seed samples from each location were malted at Montana State University in the following manner. Thirty gram samples of barley were placed into perforated plastic bottles (500 ml volume), placed into a 16 C aerated water bath, and steeped to 45% moisture (dry weight basis). At this point the samples weighed approximately 40 g. About 4 g of seed were removed from each steeping bottle and kilned at 65 C for 24 hours. These steep samples were unreplicated. The remaining 36 g of barley seed were split into three 12 g portions and placed in germination boxes between two moistened blotters. The germination boxes were placed into a germination incubator at 15 C and continuous darkness. The three replications were blocked with each tray in the incubator

representing a block. At 24, 48, 72, and 96 hours, three g samples were removed from each box and then kilned at 65 C for 24 hours.

The kilned samples were milled using the Udy Cyclone Mill (0.5 mm screen). Maltose content of the samples was determined by using the 3,5-dinitrosalicylic acid, reducing power method (Whelan, 1964 and DeHaas et al., 1978). Maltose was extracted from 0.25 g flour samples by soaking in 0.5% NaCl solution with moderate agitation at 25 C for 1 hour. The samples settled for two hours before a 1 ml aliquot of the supernatant was assayed for dissolved maltose (reducing substances). Maltose content was reported as ug maltose/g flour.

The average maltose content of Compana vs. Wapana samples taken from the steep was compared by a paired t-test since the steep samples were not replicated. F-tests were used to evaluate the effect of isotype (starch type), environment and the isotype x environment interaction at 24, 48, 72, and 96 hours of germination. Isotype was a fixed factor and environments were random factors. The treatment means for the waxy vs. nonwaxy isotypes were compared by a t-test. Due to the design of this experiment this latter test is equivalent to a paired t-test.

Correlation and regression analyses were performed for the waxy vs. nonwaxy isotypes to determine differences in the rate of maltose

production as germination time increased (Ullrich, 1978). The normal type was assigned the x- coordinate and the waxy isotype the y-coordinate. Regression coefficients (b_{yx}), correlation coefficients (r) and coefficients of determination (r^2) were calculated. The named isotype was plotted against the normal. As a reference line the normal isotype was plotted against itself to produce an r = 1 and a b_{yx} of 1.

Twelve of the paired samples used in this study were also sent to Madison Malt Laboratory (see acknowledgements) for routine analysis. The results for three characters are presented in this study: alpha-amylase (20° units), malt extract (percent), and diastatic power (degrees lintner). Means were compared by a paired t-test.

Study II: The Influence of Germination Interval Upon Maltose Production in Waxy vs. Nonwaxy Isotypes of Three Cultivars

Waxy and nonwaxy isotypes of Compana, Betzes, and Titan were grown at Bozeman, Huntley, and Moccasin, Montana in 1978 in unreplicated, paired plots. Seed samples from these plots were malted and seed were removed over time, and assayed for maltose in the same manner as described in Study I of this Chapter.

F-tests were used to measure the significance of the three main factors: genotype, isotype, and environment at 24, 48, 72, and 96 hours of germination. All factors were fixed in this study. Any

conclusions drawn from data generated by this study apply only to the three Montana locations and not to the environment in general.

A paired t-test was used to compare the means of the waxy vs. nonwaxy steep samples (cultivars were combined). T-tests were used to compare the waxy vs. nonwaxy means at 24, 48, 72, and 96 hours. Regression analysis was used to compare waxy vs. nonwaxy isotypes (cultivars are combined) as described in Study I of this chapter.

Study III: The Influence of Waxy vs. Nonwaxy Endosperm in Three Cultivars Upon Maltose Production Within Finished Malt

Waxy and nonwaxy isotypes of Compana, Titan, and Betzes, and a Klages check, were grown in eight Montana environments (see Chapter 6 for specific locations) with three replications (blocks) of each entry at each environment. The field design was a split-plot with cultivars as main plots and isotypes as subplots. This basic design was run over the eight environments. Isotype and cultivar were fixed factors and environment was random. The expected mean squares are listed in Appendix I. The basic field design was maintained throughout the malting procedure.

Grain samples from these plots were malted in the following manner. Twenty gram seed samples of each field replication were steeped to 45% moisture in an aerated 16 C water bath. The steeped

barley was transferred to germination boxes, placed between moistened blotters, and allowed to germinate at 15 C until the average length of acrospire was 2/3 - 3/4 the length of the seed. Each field replication was germinated for 74, 80, and 80 hours, for replications 1, 2, and 3, respectively. At the end of the germination period samples were kilned at 65 C for 24 hours.

The kilned samples were ground in a Udy Cyclone Mill (0.5 mm screen). Maltose content was determined as described in Study I of this chapter with the exception that the extraction solution was centrifuged at 3,000 rpm for 7 minutes in place of the 2 hour settling period.

Alpha-amylase content was determined for these samples using the BPA assay described in Chapter 3. All BPA alpha-amylase area values were converted to CBA dye release units.

Two separate analyses of variance tables were calculated for alpha-amylase and maltose content. The first analysis included only the waxy and nonwaxy pairs. In the second analysis, each isotype was considered a separate entry and the malting barley Klages was included. The purpose of this analysis was to compare the performance of the waxy isotypes with the malt industry standard. Differences between isotype or entry means were compared by a t-test.

Results and Discussion

Study I: The Influence of Germination Interval Upon Maltose Production in Waxy vs. Nonwaxy Compana

Highly significant differences were detected between the waxy and nonwaxy isotypes and among the 15 environments for maltose content at all germination times (Table 5-1). The average maltose content of the waxy isotype was significantly higher than the normal isotype after steeping and at all germination times (24, 48, 72, and 96 hours). A significant isotype x environment interaction was detected by the 24 hour germination time. This interaction can be explained by two environments in which the mean maltose content at 24 hours of germination of the waxy isotype was equal to or less than the nonwaxy isotype.

Figure 5-1 shows the regression of mean maltose content of the waxy vs. nonwaxy isotype of Compana at 0, 24, 48, 72, and 96 hours of germination. The very high r (.99**) indicates that most of the variability in maltose production across germination time can be accounted for by the waxy isotype-nonwaxy isotype association. The slope (b_{yx}) of the mutant vs. normal regression is 1.16 which is different from the unity slope (P = .10). Thus, as germination time increases, the waxy isotype increases in maltose content at a faster rate than the normal isotype.

Table 5-1. Analysis of variance for maltose produced (umoles maltose/g flour) by Compana and Wapana at four stages of micro-malting and comparison of isotype means at five stages of micro-malting.

			Mean Squares			
Source of Variance	df	Steep	24 hours	48 hours	72 hours	96 hours
Blocks	2		4719.0**	43460.0**	6404.0**	36200.0**
Isotype (I)	1		5954.0**	2395.0**	35740.0**	115500.0**
Environment (E)	14		1334.0**	2572.0**	4649.0**	6833.0**
I x E	14		307.1*	332.4	811.9	1426.0
Error	58		159.4	291.8	549.9	1896.0
Waxy ($\bar{x}_1$)		44.34	93.85	182.20	265.20	430.00
Nonwaxy ($\bar{x}_2$)		23.04	77.59	171.90	225.40	358.40
$\bar{x}_1 - \bar{x}_2$		21.30**	16.26**	10.30**	39.80**	71.60**

*, ** Significant at the 0.05 and 0.01 levels, respectively.

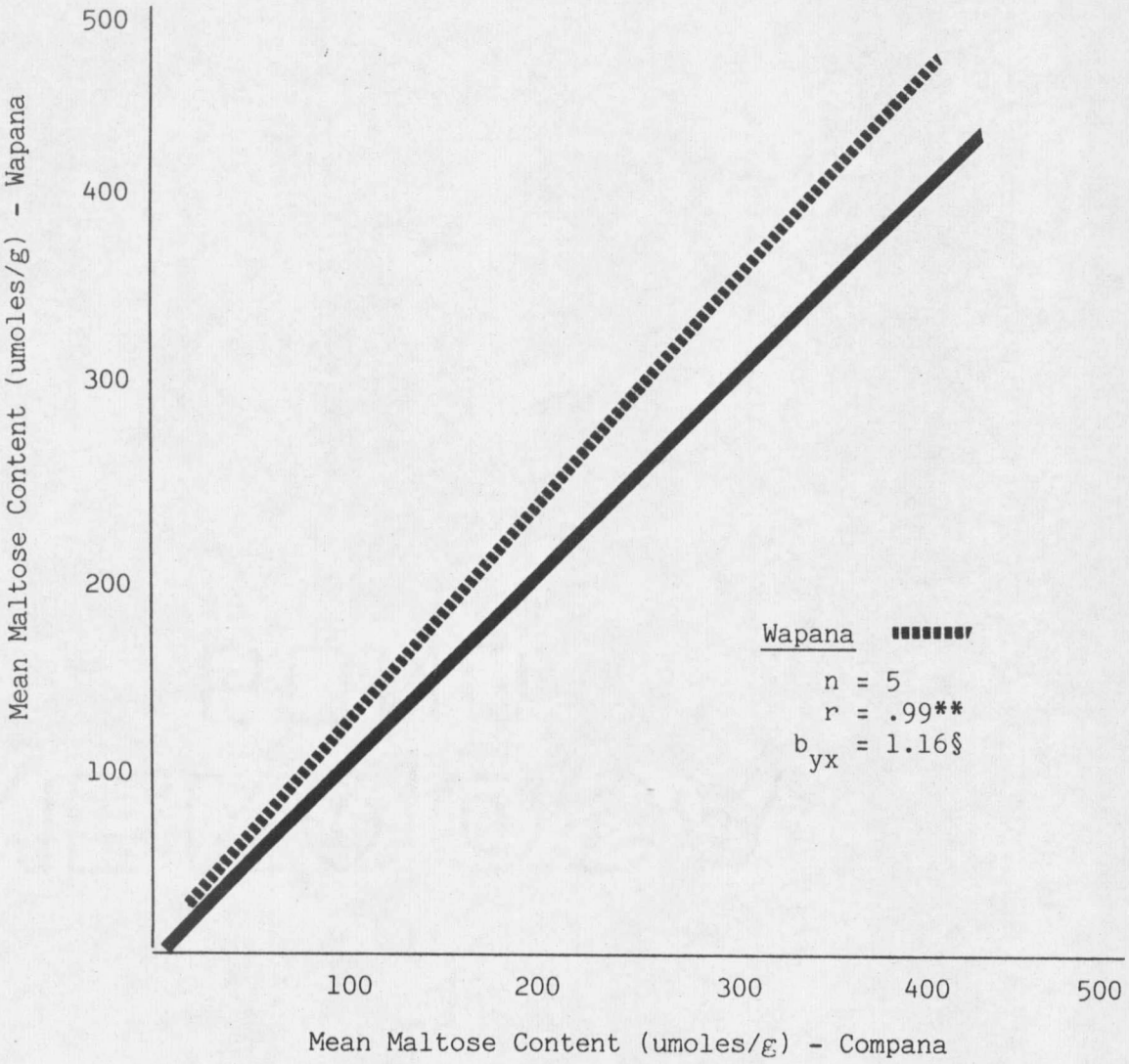

Figure 5-1. Comparison of mean starch digestibility of Wapana and Compana grown in 15 environments at 0, 24, 48, 72, and 96 hours of malting.
**Significant at the 0.01 level.
§Significantly different (P = 0.10) from a slope of 1.0.

The results of the Madison Malt Laboratory analysis are presented in Table 5-2. Malt extract percent (a measure of conversion efficiency after malting) and alpha-amylase activity were not significantly different between waxy and nonwaxy Compana. The diastatic power (D.P.) was significantly lower (P = .01) in the waxy isotype, and Table 5-2 shows that this difference is fairly consistent across environments. In only one environment does the waxy isotype exceed the nonwaxy in D.P.

Diastatic power represents the combined saccharifying power of alpha- and beta-amylase. Since the alpha-amylase activity of the two isotypes were identical, then the waxy isotype must have contained less beta-amylase than the nonwaxy. In spite of the low D.P. values, the conversion efficiency of the waxy isotype was the same as the nonwaxy. There is no simple explanation for this phenomenon. It is not uncommon to find feedback control mechanisms in living organisms based on end product inhibition which regulates enzyme production. In the case of waxy barley it is possible that less beta-amylase is produced in the developing seed since less is needed to degrade the more digestible waxy starch.

Table 5-2. Alpha-amylase activity, malt extract, and diastatic power of micro-malts as determined by the Madison Malt Laboratory for Compana-Wapana pairs grown in 12 Montana environments.

Environment	Alpha-Amylase Activity (20° units)		Malt Extract (%)		Diastatic Power (°L)	
	Compana ($\bar{x}_1$)	Wapana ($\bar{x}_2$)	Compana ($\bar{x}_1$)	Wapana ($\bar{x}_2$)	Compana ($\bar{x}_1$)	Wapana ($\bar{x}_2$)
Sidney - dryland	28.3	24.7	75.8	75.1	151	127
Creston - irrigated	23.2	20.2	73.0	72.3	177	150
Creston - dryland	29.5	29.4	70.1	64.5	190	169
Huntley - irrigated	24.9	26.7	73.8	74.4	162	127
Huntley - dryland	29.4	31.7	70.7	70.8	177	161
Fort Ellis - dryland	27.2	39.0	78.0	77.1	102	91
Fairfield - irrigated	29.1	28.3	76.4	76.3	136	117
Moccasin - dryland	42.3	42.3	68.6	69.1	161	145
Havre - dryland	23.0	24.3	70.2	70.6	147	135
Bozeman - 16 lbs nitrogen	28.5	30.1	72.4	73.1	153	186
Bozeman - 96 lbs nitrogen	33.2	30.2	73.4	72.5	186	153
Bozeman - early	30.2	28.0	74.3	73.3	173	145
$\bar{x}$	29.1	29.6	73.1	72.8	160	142
$\bar{x}_1 - \bar{x}_2$	-0.5 N.S.		0.3 N.S.		18**	

**Significant at the 0.01 level.

Study II: The Influence of Germination Interval Upon Maltose Production in Waxy vs. Nonwaxy Isotypes of Three Cultivars

The effect of isotype upon maltose production was highly significant at all germination intervals (Table 5-3). The waxy isotypes had a significantly higher mean maltose content than the nonwaxy isotypes at all germination intervals including the steep ($P = .01$).

Location had a significant effect on maltose content at all germination times except 48 hours. The effect of genotype was significant only at 24 hours of germination. Significant genotype x location interactions were observed at 24 ($P = .05$) and 72 hours ($P = .01$) of germination. These interactions were due to the fact that genotypes did not behave uniformly across low maltose to high maltose locations. This is understandable since the classification of locations by mean maltose content is partially a statistical classification in that it does not represent a definable agronomic environment.

At 48 hours germination, there was a highly significant isotype x genotype interaction. This was due to the waxy-nonwaxy pair of Compana, which had almost identical maltose content, while the combined waxy-nonwaxy pairs were significantly different.

The regression of the mean of the combined waxy isotypes vs. the mean of the nonwaxy isotypes across 0, 24, 48, 72, and 96 hours of

Table 5-3. Analysis of variance for maltose produced (umoles maltose/g flour) at four stages of micro-malting by nonwaxy-waxy pairs of three cultivars grown at three locations and comparison of isotype means at five stages of micro-malting.

		Mean Squares				
Source of Variation	df	Steep	24 hours	48 hours	72 hours	96 hours
Blocks	2		1305.5**	335.3	4917.0**	8200.0**
Isotype (I)	1		4078.0**	4461.9**	5020.0**	48030.0**
Cultivar (C)	2		518.9*	234.6	1083.0	608.5
Location (L)	2		1127.0**	402.2	3712.5**	85900.0**
I x L	2		122.4	51.4	786.2	3100.0
I x C	2		224.0	1572.6**	86.7	3313.0
C x L	4		508.6*	233.5	1869.4**	2548.3
I x C x L	4		25.4	126.3	679.3	1097.0
Error	34		138.6	260.6	426.3	1456.5
Waxy ($\bar{x}_1$)		42.89	82.13	148.57	226.10	395.40
Nonwaxy ($\bar{x}_2$)		23.54	64.75	130.39	206.80	335.70
$\bar{x}_1 - \bar{x}_2$		19.35**	17.38**	18.18**	19.30**	59.70**

*, ** Significant at the 0.05 and 0.01 levels, respectively.

germination (Figure 5-2) had an r = .99**, and a b_{yx} = 1.12 which was different from the unity slope (P = .10). Thus, as in the previous study, the waxy and nonwaxy isotypes behaved in a highly correlated manner across germination times. Waxy types, however, had a greater b_{yx} value than nonwaxy isotypes indicating the rate of maltose accumulation was faster in waxy isotypes as germination time is increased.

Study III: The Influence of Waxy vs. Nonwaxy Endosperm in Three Cultivars Upon Maltose Production Within Finished Malt

Highly significant differences in maltose production were detected between isotypes (Table 5-4). Genotype and environment were not significant sources of variation in this study. The mean maltose content of waxy isotypes was significantly higher than that of nonwaxy types (3.24 umoles maltose/gram flour vs. 2.68 umoles maltose/gram flour; P = .001).

A significant genotype x isotype interaction was observed. This was due to differences in the magnitude of response by the three cultivars when the waxy gene was incorporated. Average maltose content increased in all three backgrounds, but not to the same degree. Titan had an increase in maltose content of 28%; Compana 22%; and Betzes 13%.

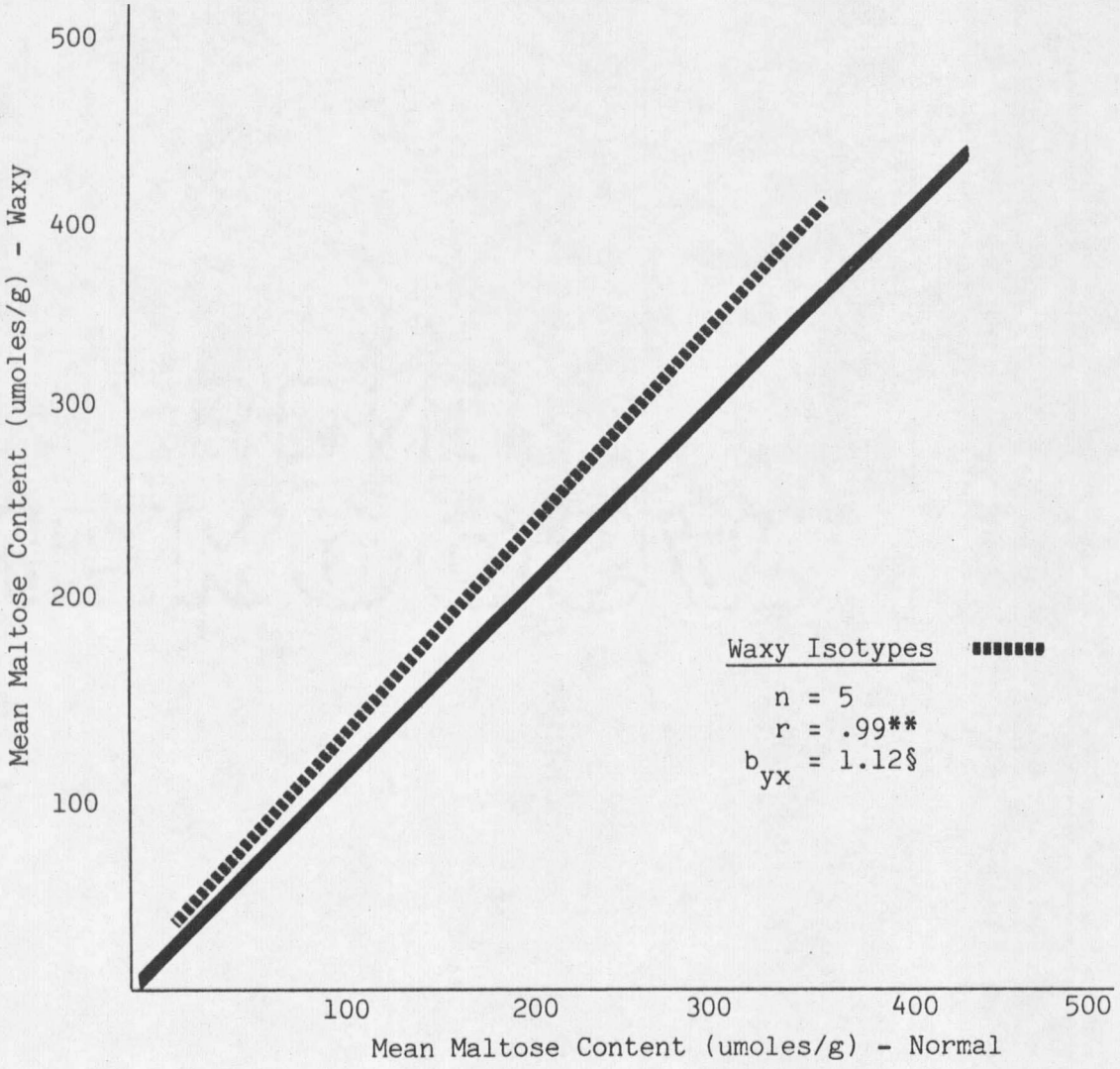

Figure 5-2. Comparison of mean starch digestibility of waxy and non-waxy isotypes of Compana, Titan, and Betzes grown at three locations at 0, 24, 48, 72, and 96 hours of malting.
**Significant at the 0.01 level.
§Significantly different (P = 0.10) from a slope of 1.0.

Table 5-4. Analysis of variance for maltose produced (umoles maltose/g flour) in finished malt of nonwaxy-waxy pairs in three cultivars grown in eight environments.

Source of Variance	df	SS	MS	F
Environment (E)	7	9.667	1.381	1.240
Blocks/Environment (B/E)	16	17.820	1.114	
Cultivar (C)	2	0.009	0.005	0.029
C x E	14	2.400	0.171	1.554
(B/E) x C	32	3.520	0.110	
Isotype (I)	1	11.440	11.440	228.800**
I x E	7	0.351	0.050	0.781
I x C	2	0.874	0.437	3.833*
I x C x E	14	1.601	0.114	1.781
((B/E) x I) + ((B/E) x C x I)	48	3.078	0.064	

*, **Significant at the 0.05 and 0.01 levels, respectively.

No single main effect (isotype, environment, or genotype) had a significant effect on alpha-amylase produced during malting (Table 5-5). The environment x genotype interaction was significant. Genotypes did not behave uniformly across low alpha-amylase to high alpha-amylase environments. This is understandable since the classification of environments by mean alpha-amylase activity is partially a statistical classification in that it does not represent a definable agronomic environment.

To compare the performance of the waxy and nonwaxy isotypes to an outstanding malting variety a second analysis was performed which included the variety Klages. The individual waxy and nonwaxy isotypes were treated as individual entries in this analysis.

Highly significant differences in maltose content was detected among entries (Table 5-6). Differences were not detected among environments. Figure 5-3 shows that Klages was significantly higher in mean maltose content than all three of the normal isotypes. This is expected, since Klages is presently the only two-row malting barley with high enzyme activity. The waxy isotypes were higher than (Titan) or equal to (Betzes, Compana) Klages in mean maltose content.

Significant differences in alpha-amylase activity were detected among entries (Table 5-7). Figure 5-4 shows that Klages had a higher mean alpha-amylase activity than any of the waxy and nonwaxy isotypes.

Table 5-5. Analysis of variance for BPA alpha-amylase activity (ug dye released/mg flour/min at 65 C) produced in finished malt of nonwaxy-waxy pairs in three cultivars grown in eight environments.

Source of Variance	df	SS	MS	F
Environment (E)	7	574.20	82.03	2.27
Blocks/Environment (B/E)	16	587.60	36.16	
Cultivar (C)	2	13.92	6.96	0.26
C x E	14	377.40	26.96	2.21*
(B/E) x C	32	389.80	12.18	
Isotype (I)	1	20.65	20.65	1.79
I x E	7	80.95	11.56	0.67
I x C	2	56.24	28.12	1.49
I x C x E	14	264.30	18.87	1.10
((B/E) x I) + ((B/E) x C x I)	48	826.60	17.22	

*Significant at the 0.05 level.

Table 5-6. Analysis of variance for maltose produced (umoles maltose/g flour) in finished malt of seven cultivars in eight environments.

Source of Variance	df	SS	MS	F
Environment (E)	7	10.86	1.55	1.24
Blocks/Environment	16	20.05	1.25	
Cultivar (C)	6	12.85	2.14	15.29**
C x E	42	5.75	0.14	1.75*
Error	46	7.82	0.08	

*, **Significant at the 0.05 and 0.01 levels, respectively.

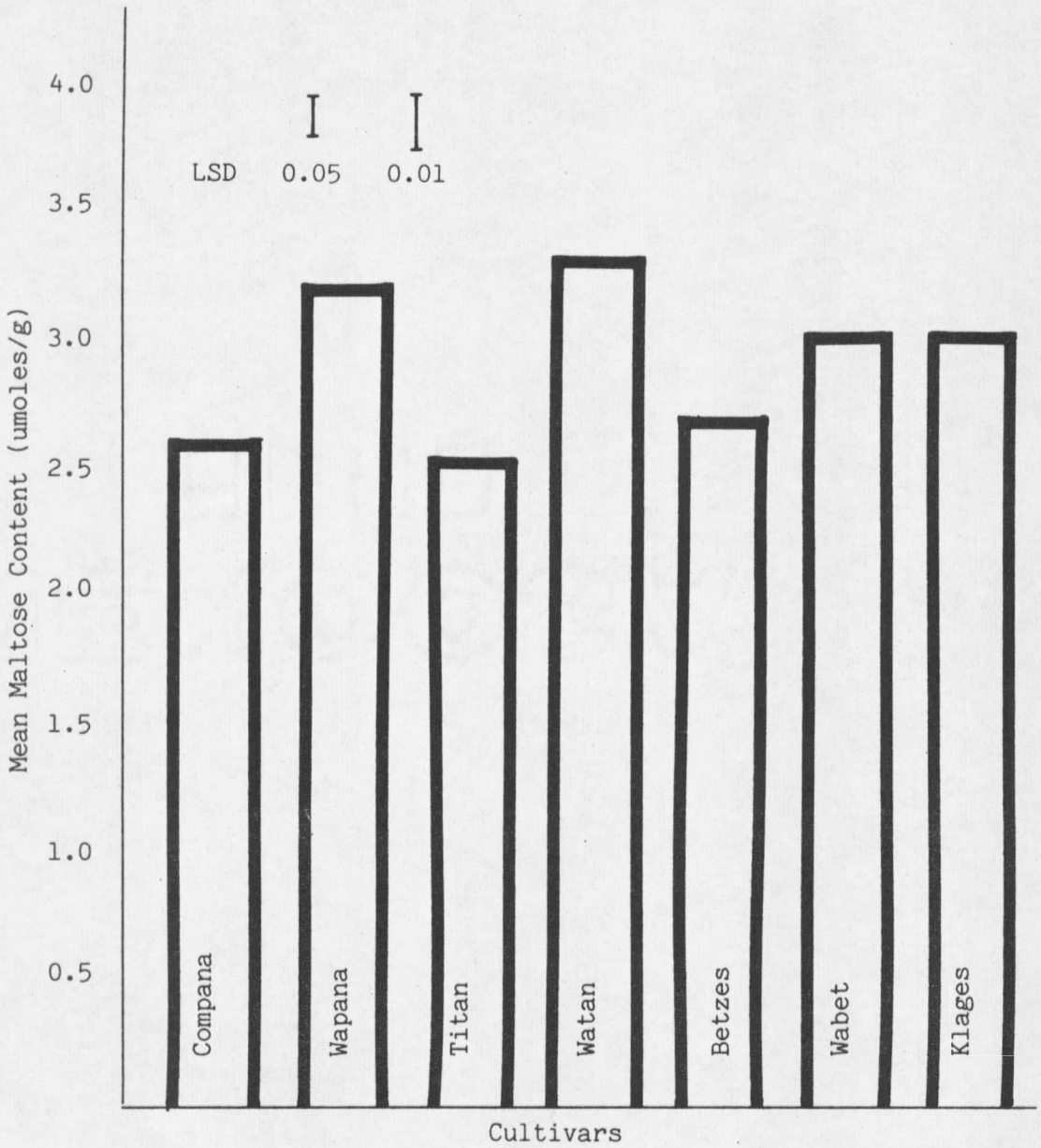

Figure 5-3. Mean maltose content in finished malt of nonwaxy-waxy pairs of Compana, Titan, and Betzes and a Klages check grown in eight environments.

Table 5-7. Analysis of variance for BPA alpha-amylase activity (ug dye released/mg flour/min at 65 C) produced in finished malt of seven cultivars grown in eight environments.

Source of Variance	df	SS	MS	F
Environment (E)	7	523.40	74.77	1.59
Blocks/Environment	16	751.50	46.96	
Cultivar (C)	6	482.70	80.45	3.13*
C x E	42	1078.00	25.66	1.21
Error	96	2040.00	21.25	

*Significant at the 0.05 level.

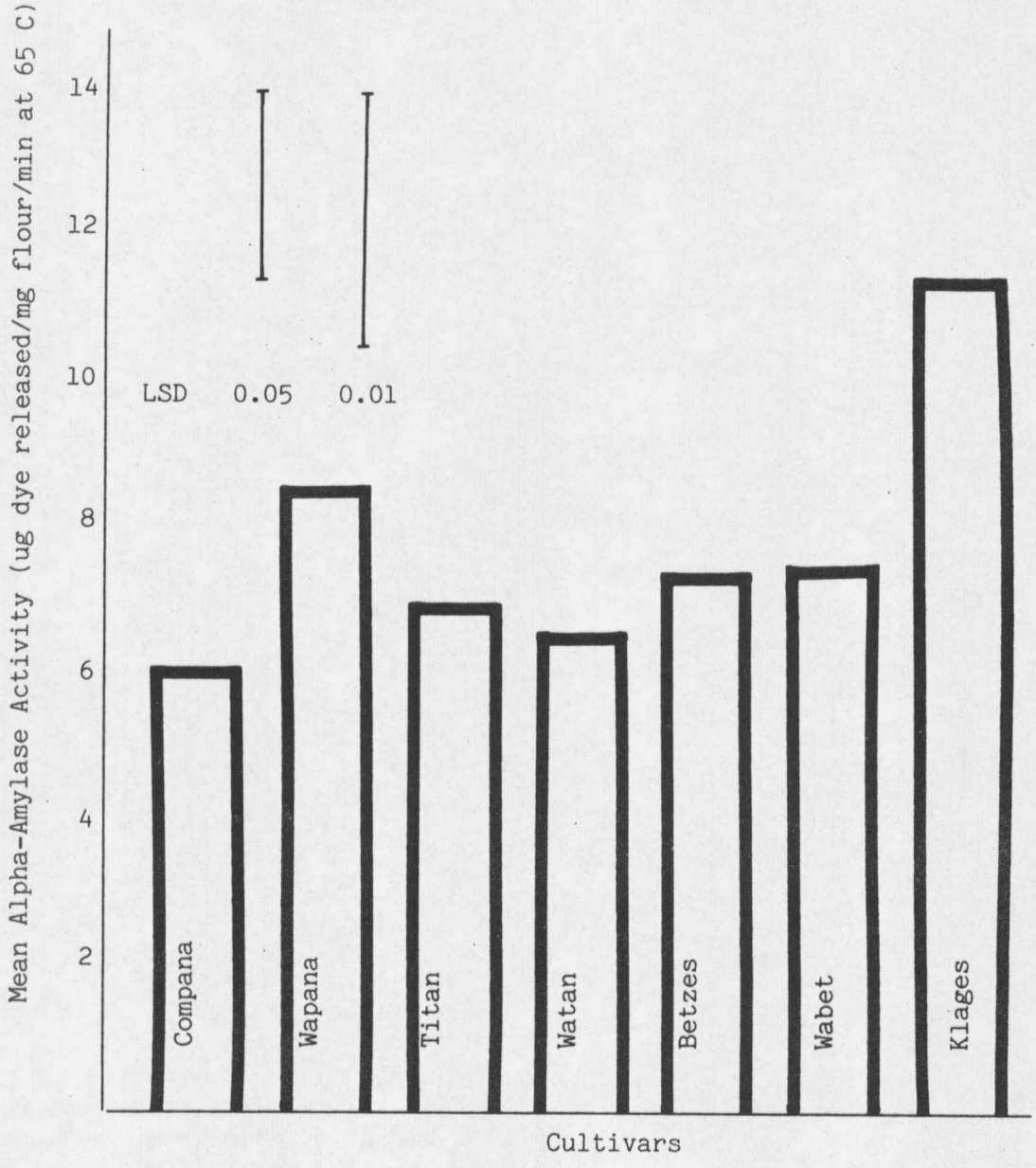

Figure 5-4. Mean alpha-amylase activity in finished malt of nonwaxy-waxy pairs of Compana, Titan, and Betzes and a Klages check grown in eight environments.

It is evident from these results that the waxy isotypes convert starch to reducing substances at a rate equal to or greater than the industry standard, Klages. This conversion is affected using smaller amounts of alpha-amylase and probably beta-amylase. The purpose of malting is primarily to produce enzymes. But the brewer, interested in converting starch to sugar for fermentation, should find useful the ability of a waxy isotype to convert faster with less enzyme.

Summary

The introduction of the waxy gene into three barley genotypes resulted in a highly significant ($P = .01$) increase in maltose or reducing substances produced at all stages of the malting process (after steeping, 24, 48, 72, and 96 hours). The rate of maltose production over germination time was greater ($P = .10$) for waxy isotypes when compared to their normal counterparts.

A genotype x isotype interaction was observed for maltose produced in finished malt. The Betzes genotype did not increase in maltose production to the same extent as Compana and Titan when the waxy gene was incorporated.

When alpha-amylase and extract measurements were made for waxy and nonwaxy seed samples of Compana, no significant difference was observed. However, a significant reduction in diastatic power was

observed (P = .01) in the waxy isotype indicating that it contained less beta-amylase than the nonwaxy isotype.

Alpha-amylase measurements were made on waxy vs. nonwaxy isotypes of three cultivars which were malted. No significant difference in alpha-amylase activity was detected between isotypes or genotypes.

The results indicate that upon malting waxy barley, it produces more reducing substances (maltose) than nonwaxy types while producing the same levels of alpha-amylase and, for at least one variety, lower levels of beta-amylase. This phenomenon can be explained by the extremely digestible nature of the waxy starch.

CHAPTER 6

THE EFFECT OF THE WAXY, SHORT-AWN, AND HULLESS GENES UPON SEED QUALITY CHARACTERISTICS IN THREE CULTIVARS

Introduction

The purpose of this study was to determine what effect the waxy (*wxwx*), short-awn (*lk2lk2*), and hulless (*nn*) genes, singly and in combination, have upon kernel weight, percent protein, percent extract, beta-glucan viscosity, and alpha-amylase activity in ripe, nongerminated barley seed.

The main impetus for this study was the excessive viscosity noted for warm water suspensions of Washonupana barley flour being processed into syrup. The viscosity was attributed to beta-glucan content of the barley flour. Thus, it seemed important to characterize the three genes which were involved in the development of Washonupana with regard to their influence on beta-glucan content as well as the other mentioned characteristics. These other characteristics (percent protein, percent extract, kernel weight, and alpha-amylase activity) may become important industrial considerations in the future and may have a direct or indirect effect on beta-glucan content in the seed.

Materials and Methods

Isotypes

All of the available waxy endosperm, short-awn, hulless isotypes of the varieties Titan, Betzes, and Compana were used for this study. The naming and derivation of the isotypes was discussed in Chapter 2. The isotypes available in Compana were: Nupana, Shopana, Wapana, Shonupana, Wanupana, and Washonupana. Washopana was not available. The isotypes available in Betzes were: 'Nubet', 'Shobet', 'Wabet', 'Shonubet', and 'Wanubet'. 'Washobet' and 'Washonubet' were not available. The isotypes available in Titan were: 'Nutan', 'Shotan', 'Watan', 'Shonutan', and 'Wanutan'. 'Washotan' and 'Washonutan' were not available. An abbreviated genetic symbol (Table 6-1) is used when discussing the various isotypes.

Commercial Check Cultivars

Five cultivars were included in this experiment as commercial checks. All were nonwaxy, long-awn, and covered cultivars. Klages (C.I. 15478) is a two-row malting barley noted for high enzyme production and considered the standard of the industry. 'Piroline' (C.I. 9558) is a two-row malt/feed barley. Shabet (C.I. 13827) is a two-row malting barley (basically a Betzes type in which shatter resistance has been incorporated). 'Summit' is a two-row feed

Table 6-1. Abbreviated genetic symbols of the waxy, short-awn, hulless isotypes.

Isotype Name Code	Genetic Description	Genetic Abbreviation
wa-	waxy	wx
nu-	hulless	n
sho-	short-awn	lk2
shonu-	short-awn, hulless	lk2 n
wanu-	waxy, hulless	wx n
washo-	waxy, short-awn	wx lk2
washonu-	waxy, short-awn, hulless	wx lk2 n
normal	nonwaxy, long-awn, covered	Wx Lk2 N

barley. 'Gus' is a six-row feed barley noted for short stiff straw, strictly designed for irrigated production.

Experimental Design and Analysis

The isotypes and cultivars (genotypes) were grown in single row plots at eight Montana locations (environments). Row widths at all environments were 0.254 m, and lengths varied from 2.44m to 4.88m. The basic design of the experiment was a split-plot in which genotypes were main plots and isotypes were subplots. The split-plot was contained in three blocks at each environment. The environments were those that existed at Huntley (irrigated and dryland), Kalispell (irrigated and dryland), Moccasin (dryland), Sidney (irrigated), Bozeman (irrigated), and Ft. Ellis (dryland) in 1979. A diagram of the experimental design is contained in Appendix II.

The first analysis (combined) of the experiment includes the Wx Lk2 N, wx, n, lk2, wx n, and lk2 n isotypes of all three genotypes at the eight environments. The factors, genotype and isotype, were fixed, and environment was random. The significance of each factor and their interactions were determined with an F-test. The expected mean squares are listed in Appendix I. Comparisons of means was done with a t-test. For isotypes, five planned, nonorthogonal comparisons were made for all response variables: 1) Wx Lk2 N vs. wx; 2) Wx Lk2

N vs. lk2; 3) Wx Lk2 N vs. n; 4) n vs. wx n; and 5) n vs. lk2 n. The comparison of wx vs. wx n was made for beta-glucan viscosity. In addition, isotypes were ordered according to kernel weight, then each isotype compared to the next higher and next lower isotypes.

Some unplanned comparisons were made. This practice is appropriate when the F-test is significant (Fisher, 1966). Genotype means were compared for differences among them. Since environments were considered random, no attempt was made to analyze individual environment means.

The second analysis of this experiment was performed in order to examine the isotype response within a genotype. The Wx Lk2 N, wx, n, lk2, wx n, and lk2 n isotypes of each genotype at the eight environments were included in three separate analyses. In addition, the wx lk2 n isotype was included in the Compana analysis. As with the first analysis, isotypes were fixed and environments random. All comparisons among isotypes were made as described above. The comparison of lk2 n vs. wx lk2 n in Compana was made for all response variables. The comparison of wx n vs. wx lk2 n in Compana was made for beta-glucan viscosity.

The third analysis of this experiment included the five commercial check cultivars (genotypes). Genotypes were fixed and environments random. No planned comparisons were made. The main

function of the checks was to provide a general comparison of how isotypes compare to common commercial cultivars.

Regression analysis was used to compare mean beta-glucan viscosity vs. mean kernel weight of nonwaxy isotypes. The mean kernel weights ($\bar{x}$) of the individual waxy isotypes (i) across genotypes (j) were inserted into a prediction equation, $\hat{Y} = \bar{\bar{Y}} + b\bar{x}_{i.}$, and a $\hat{Y}$, predicted mean beta-glucan, calculated. The deviation between $\hat{Y}$ and $\bar{y}$ was tested using the calculated standard error for the prediction of $\bar{y}$ for a new member of the x-array (Snedecor and Cochran, 1967). This procedure was also used for the analysis of isotypes within genotypes ($\hat{Y} = \bar{\bar{Y}} + b\bar{x}_{ij}$).

Regression analysis was also used to relate the mean percent protein of isotypes to the mean alpha-amylase activity of isotypes. In order to determine a significant relationship, r values were calculated. This procedure was used for the analysis of isotypes across genotypes, and within genotypes. Regression coefficients for isotypes within genotypes were compared using a method described by Snedecor and Cochran (1967).

Quality Determinations

Kernel Weight--Kernel weights were determined by weighing 300 whole undamaged seed. This character is reported as mg/seed.

Beta-Glucan Viscosity--Viscosities were determined using a method described by Bendelow (personal communication). Samples of 300 whole undamaged seed were ground in a Udy Cyclone Mill using a 0.5 mm screen. Flour samples of 2.5 g were weighed and placed into a 125 ml Erlenmeyer flasks and dispersed in 50 ml of sodium carbonate-bicarbonate buffer (ph 10). At this pH, all glucanase activity is halted. Samples were mixed thoroughly with care given to dispersing all clumped flour. Flasks were plugged and placed in a shaking water bath at 45 C for 50 minutes. The samples were removed and cooled to 25 C, then centrifuged for 7 minutes at 3000 RPM. Samples were placed in a 25 C water bath until measured. Viscosity was measured by using a Brookfield viscometer with a ULV adaptor. Sample supernatant was poured into the ULV adaptor cup and placed in a 25 C water bath. Viscosity was then measured as centipose units/sec at 25 C (cps, 25 C). The resultant viscosity is termed "beta-glucan viscosity."

The determinations were done on groups of 24 samples with each group of 24 representing a field replication within a location.

The above assay is described as a screening technique for beta-glucan content of barley (Bendelow, personal communication). However, MacGregor (unpublished data) found that the increased viscosity observed for Washonupana was due to an actual increase in the amount of soluble beta-glucan. He observed that actual percent beta-glucan

almost doubled in one Washonupana sample when compared to the normal. This was a preliminary examination with unreplicated samples.

These observations suggest that this assay can be used as an indicator of relative beta-glucan content of barley samples. However, this assay does measure directly the viscosity produced when a barley flour sample is mixed with warm water. The phenomenon, whatever the cause, is the factor of interest to syrup and other industrial processors of nongerminated seed.

Percent Protein--Percent grain protein was measured using the Technicon Infraalyzer 400 (NIR). A program internal to the machine will predict percent protein on the basis of infrared reflectance of selected frequency bands sensitive to protein. Samples were prepared for analysis by grinding in a Udy Cyclone Mill (0.5 mm screen).

Percent Extract--Starch measurements were not obtained directly. McGuire (1981) described a method predicting malt extract using the Technicon Infraalyzer 400. The method entailed the use of two-infrared frequencies, one specific for protein reflectance, and the other for starch. A prediction equation was calculated to predict potential malt extract (McGuire, personal communication). McGuire has noted that this calibration is probably more sensitive in predicting the starch to protein ratio than actual malt extract.

This determination was made on all samples in this study to provide a possible indication of the relative difference in starch accumulation among isotypes. No absolute value for starch content can be ascertained from this data.

Alpha-Amylase Activity--Alpha-amylase was determined for all samples using the BPA assay described in Chapter 3. Alpha-amylase area is converted to ng CBA dye release/mg flour/min at 65 C.

The purpose of the alpha-amylase study was to determine enzyme content in sound seed. The Kalispell irrigated environment showed sprout damage. This environment was deleted from the alpha-amylase analysis.

Results and Discussion

Analysis of Variance

Highly significant differenced (P = .01) were detected among environments, genotypes, and isotypes (Table 6-2) for the response variables kernel weight, beta-glucan viscosity, percent protein, and percent extract. Significant differences (P = .05) were detected among environments, genotypes, and isotypes for alpha-amylase activity.

Only the two-factor interactions were significant for kernel weight. All of the interactions were highly significant for beta-glucan viscosity. There were no significant interactions for percent

Table 6-2. Analysis of variance for kernel weight, beta-glucan viscosity, percent protein percent extract, and alpha-amylase activity for six isotypes across three genotypes.

Source of Variance	df	Mean Square: Kernel Weight (mg)	Mean Square: Beta-Glucan Viscosity (CPS at 25 C)	Mean Square: Protein (%)	Mean Square: Extract (%)	df	Mean Square: Alpha-Amylase Activity (ng dye released/ mg flour/min at 65 C)
Environment (E)	7	849.90**	809.90**	61.71**	85.47**	6	1375.00*
Blocks/Environment (B/E)	16	14.21	43.35	1.27	1.84	14	359.90
Genotypes (G)	2	8036.00**	1326.00**	31.82**	49.28**	2	4479.00*
G x E	14	77.39**	144.30**	1.94	2.96	12	1083.00**
(B/E) x G	32	13.43	15.41	1.04	1.52	28	338.80
Isotype (I)	5	447.60**	871.60**	11.18**	28.25**	5	358.20*
I x E	35	4.44*	99.68**	0.46	1.08*	30	121.80
I x G	10	26.99**	147.50**	0.58	1.08	10	198.30
I x G x E	70	3.97	43.38**	0.36	0.68	60	133.50
((B/E) x I) + ((B/E) x G x I)	240	2.99	7.86	0.27	0.45	210	108.60

*, **Significant at the 0.05 and 0.01 levels, respectively.

protein. For percent extract, only the environment x isotype interaction was significant. And, finally, for alpha-amylase, the environment x genotype interaction was highly significant. The significant interactions for all response variables were analyzed by regression analysis and discussed in Chapter 7.

The individual genotype analyses in Table 6-3 showed that both main effects, environment and isotype, were highly significant (P = .01) within Compana, Titan, and Betzes for kernel weight, beta-glucan viscosity, percent protein, and percent extract. Each genotype behaved differently with respect to alpha-amylase activity. Compana had a significant (P = .01) isotype effect but no environment effect. Titan (P = .05) and Betzes (P = .01) both had a significant environment effect but no isotype effect. The environment x isotype interactions within genotypes are discussed and analyzed in Chapter 7.

The analysis of the commercial cultivars indicates that environment had a highly significant (P = .01) effect on all of the response variables. Genotype was a highly significant (P = .01) source of variation for all of the response variables except protein.

Table 6-3. Analysis of variance for kernel weight, beta-glucan viscosity, percent protein, percent extract, and alpha-amylase activity of five commercial cultivars, seven isotypes within Compana, and six isotypes within Titan and Betzes.

Source of Variance	df	Mean Square: Kernel Weight (mg)	Mean Square: Beta-Glucan Viscosity (CPS at 25 C)	Mean Square: Protein (%)	Mean Square: Extract (%)	df	Mean Square: Alpha-Amylase Activity (ng dye released/mg flour/min at 65 C)
Commercial Cultivars							
Environment (E)	7	283.00**	21.32**	19.19**	25.92**	6	340.90**
Blocks/Environment (B/E)	16	9.60	3.56	1.15	1.73	14	39.66
Cultivar (C)	4	215.50**	29.33**	2.08	5.97**	4	502.00**
C x E	28	10.59*	2.55**	4.01**	0.78	24	47.78
(B/E) x C	64	6.06	1.27	0.32	0.54	56	37.25
Compana							
Environment (E)	7	566.90**	199.70**	30.06**	39.78**	6	66.22
Blocks/Environment (B/E)	16	23.89	25.47	2.10	2.71	14	111.51

Table 6-3 (Continued)

Source of Variance	df	Mean Square: Kernel Weight (mg)	Beta-Glucan Viscosity (CPS at 25 C)	Protein (%)	Extract (%)	df	Mean Square: Alpha-Amylase Activity (ng dye released/mg flour/min at 65 C)
Isotype (I)	6	410.50**	599.20**	4.05**	13.38**	6	116.30**
I x E	42	6.44*	23.00*	0.55	1.07	36	28.13*
(B/E) x I	96	4.21	10.01	0.39	0.65	84	16.38
Titan							
Environment (E)	7	97.23**	189.10**	17.92**	27.01**	6	3254.00*
Blocks/Environment (B/E)	16	7.19	10.94	0.53	1.04	14	898.50
Isotype (I)	5	133.60**	114.90**	4.77**	9.21**	5	581.30
I x E	35	2.35	12.80**	0.31*	0.71**	30	324.80
(B/E) x I	80	1.51	4.55	0.14	0.25	70	284.00
Betzes							
Environment (E)	7	400.50**	807.40**	20.38**	29.40**	6	214.10**
Blocks/Environment (B/E)	16	11.61	56.46	1.27	1.98	14	40.16

Table 6-3 (Continued)

Source of Variance	df	Mean Square				df	Mean Square
		Kernel Weight (mg)	Beta-Glucan Viscosity (CPS at 25 C)	Protein (%)	Extract (%)		Alpha-Amylase Activity (ng dye released/mg flour/min at 65 C
Isotype (I)	5	97.42**	923.80**	3.01**	6.25**	5	34.01
I x E	35	4.55*	166.70**	0.36	0.64	30	37.79
(B/E) x I	80	2.69	16.61	0.31	0.46	70	24.76

*, **Significant at the 0.05 and 0.01 levels, respectively.

Comparison of Means

Kernel Weight--A comparison of isotype means (across genotypes) from the combined analysis indicated that kernel weights were significantly (P = .01) reduced for all single isogenics when compared to the normal isotype (Wx Lk2 N vs. wx; Wx Lk2 N vs. lk2; Wx Lk2 N vs. n). Kernel weights were also significantly reduced for the double isogenics when compared to the hulless isotype (n vs. wx n, P = .05; and, n vs. lk2 n, P = .01).

The isotypes are ordered in Figure 6-1 from highest to lowest kernel weight. The order is Wx Lkw N > wx > lk2 > n > wx n > lk2 n. Each isotype was significantly different from the isotype preceding it and following it (P = .05 or .01). Kernel weight reductions had been described in barley for the short-awn, and hulless isogenics of barley (Schaller, 1972; Eslick, 1979; and McGuire and Hockett, 1981). In corn, Creech (1968) reported significantly less dry matter accumulation in waxy types.

Genotype means (across isotypes) for kernel weight (Figure 6-1) were significantly (P = .01) different from each other. Compana, the heaviest, is a two-row barley noted for large kernels and high kernel weights. Betzes, the next largest, is a two-row barley noted for a moderate sized kernel. Titan, the smallest, as a typical six-row

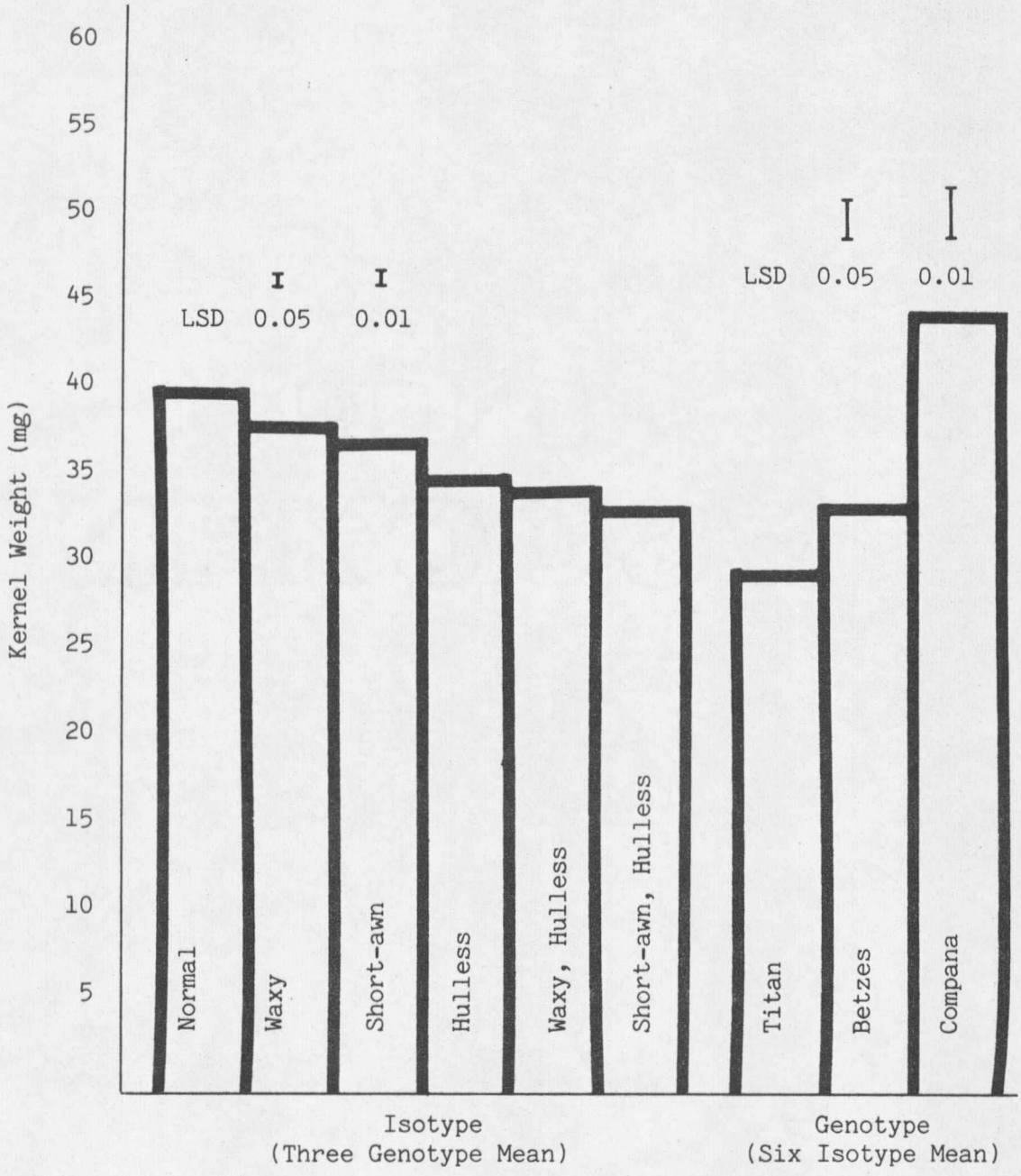

Figure 6-1. Mean kernel weight of six isotypes (across three genotypes) and three genotypes (across six isotypes) grown in eight environments.

barley, produces smaller seed and lower kernel weights than most two-row cultivars.

The reduction in kernel weight observed across genotypes as the waxy, short-awn, and hulless genes were introduced, was also observed within genotypes (Figure 6-2). Some differences were observed (significant isotype x genotype interaction, Table 6-2). The waxy gene resulted in kernel weight reductions, but they were not significantly different from the normal or hulless types in Betzes and Titan. In Compana, the differences were significant (P = .01). The order of kernel weight reduction observed in the combined analysis was maintained within Titan (Titan > Watan > Shotan > Nutan > Wanutan > Shonutan) and Betzes (Betzes > Wabet > Shobet > Nubet > Wanubet > Shonubet). Within the Compana background the order was not maintained: Compana > Wapana > Shopana > Nupana > Shonupana > Wanupana. The double isogenics Wanupana and Shonupana switched order.

The mean for the triple isogenic, Washonupana, resulted in a significant (P = .01) decrease in kernel weight when compared to Wanupana. Washonupana kernel weight was reduced 25% as compared to Compana.

For the remainder of the studies in this Chapter, the isotypes are ordered on bar graphs according to the kernel weight order in Figure 6-1.

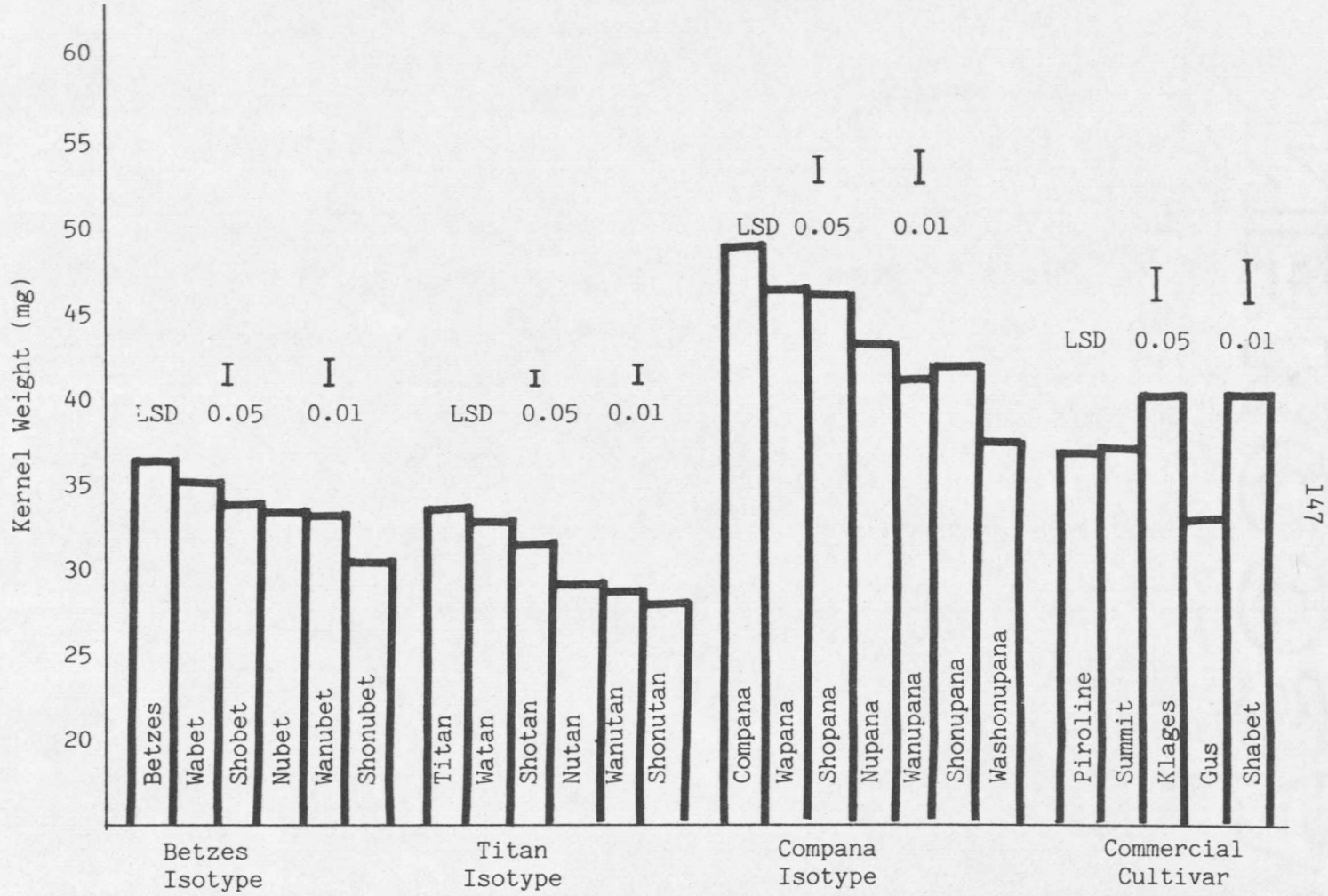

Figure 6-2. Mean kernel weight of six Betzes and Titan isotypes, seven Compana isotypes, and five commercial cultivars grown in eight environments.

Beta-Glucan Viscosity--Two observations were made by comparing isotype means (across genotypes) in Figure 6-3. First, beta-glucan viscosity reacted oppositely to kernel weights as the waxy, short-awn, and hulless genes were added. It increased as kernel weights decreased. Second, the only significant differences among isotypes was between the waxy isotypes and their normal counterparts (Wx Lk2 N vs. wx, P = .05; n vs. wx n, P = .01), and within waxy isotypes between the covered and hulless isogenes (wx vs. wx n, P = .01).

With respect to genotype differences, Betzes was significantly (P = .01) higher in beta-glucan viscosity than both Compana and Titan. Compana and Titan were similar.

The isotype x genotype interaction was highly significant. The isotype bar graph profiles for Compana and Betzes (Figure 6-4) were similar to the isotype profile in Figure 6-3. Titan had a different profile because the waxy isotypes did not have as large an increase in viscosity as in Compana and Betzes. This resulted in Shonutan having a higher mean viscosity than Watan.

The wx isotype was elevated in beta-glucan viscosity above the normal in all genotypes, but not significantly. The wx n isotype had significantly (P = .01) higher viscosities than the n isotype, and the wx isotype, in all three genotypes. Within Compana, the wx lk2 n isotype had a significantly (P = .01) higher beta-glucan

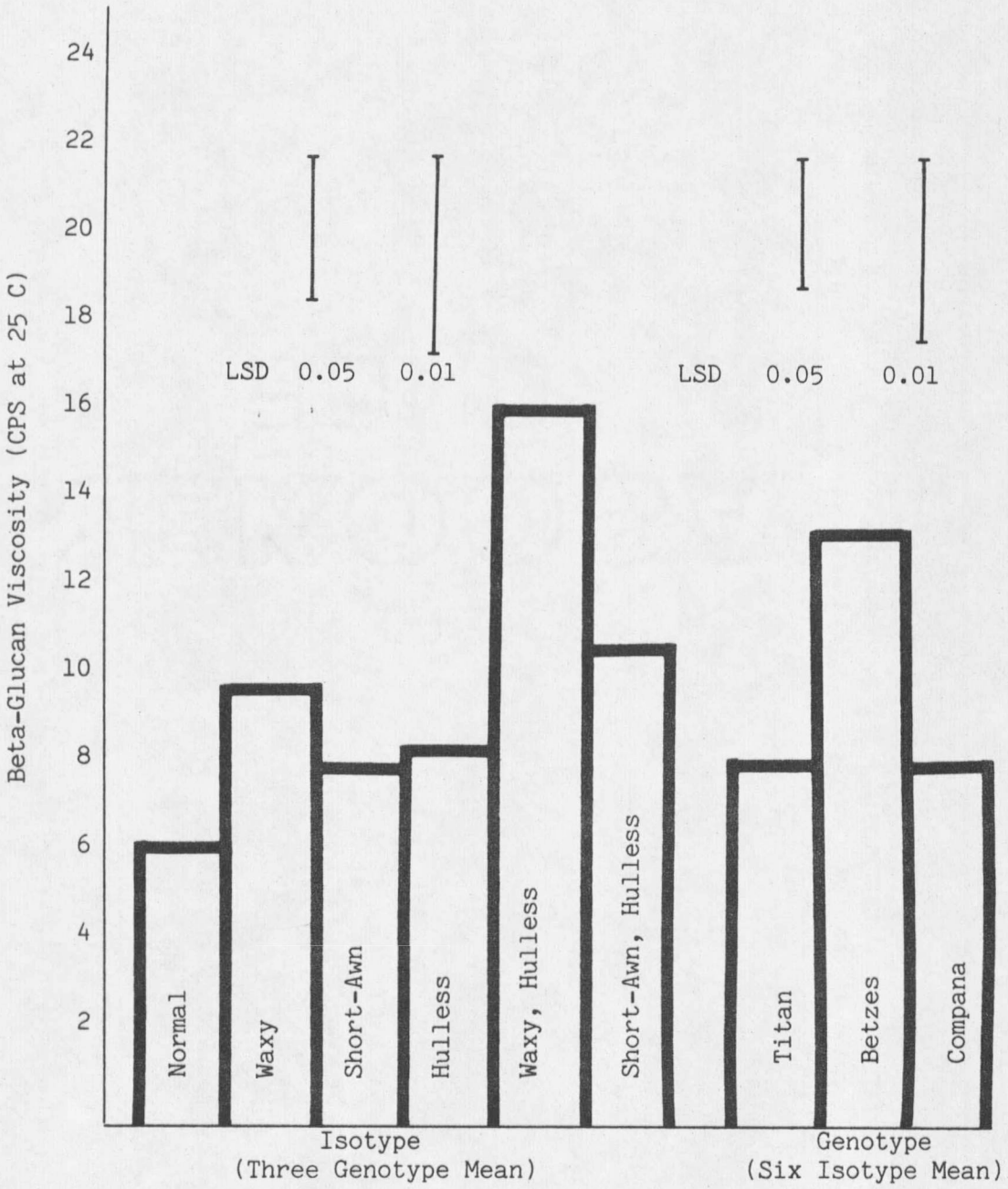

Figure 6-3. Mean beta-glucan viscosity of six isotypes (across three genotypes) and three genotypes (across six isotypes grown) in eight environments.

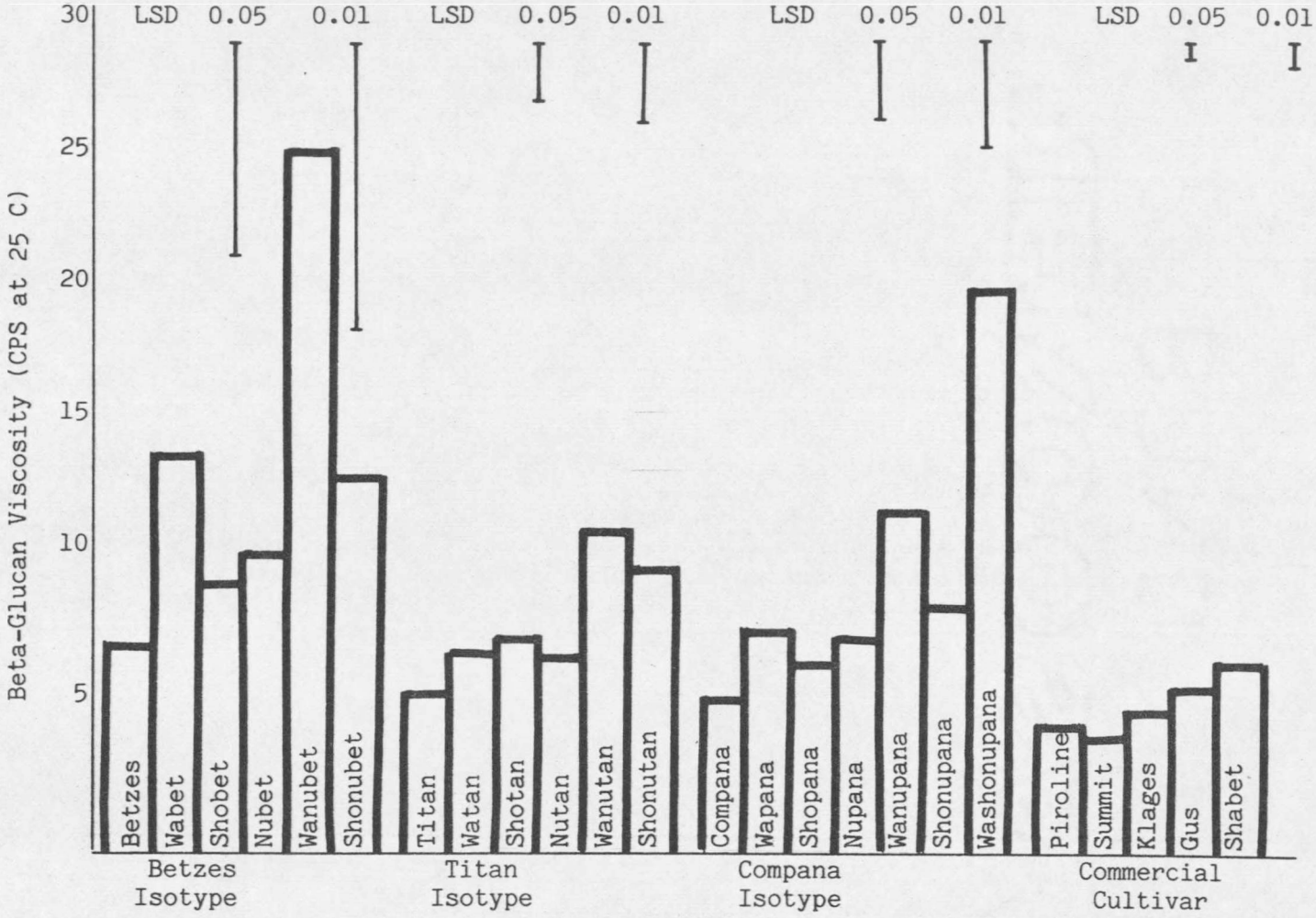

Figure 6-4. Mean beta-glucan viscosity of six Betzes and Titan isotypes, seven Compana isotypes, and five commercial cultivars grown in eight environments.

viscosity than the wx n isotype.

As beta-glucan content increases the associated viscosity of a hot water extract increases exponentially. This may explain the high error term associated with this assay. Figure 6-4 shows that the LSD bars are not equal for all groups of samples. Betzes was greater than Titan and Compana, and the commercial checks were lowest. This indicates that as the average beta-glucan viscosity increases, so does the associated variance. This was one reason individual genotype analyses were performed.

The magnitude of the problem that viscosity presents to industrial processing can be illustrated by Figure 6-4. Note the extent to which the viscosity of Washonupana is elevated above any of the commercial checks (3-4 times greater viscosity).

The overall conclusion that was obtained from this study was that as kernel weights were reduced by short-awn or hulless-seed genes, beta-glucan viscosity increased proportionately. The addition of the waxy gene caused an increase in beta-glucan viscosity disproportionate to its kernel weight reduction.

The isogenic background of the waxy isotype had a significant effect on this increase. For all genotypes, the wx n isotype was significantly higher in viscosity than the wx isotype. In Compana

where the wx lk2 n isotype was available, there were significant differences in beta-glucan viscosity between all waxy isotypes such that, wx lk2 n > wx n > wx.

Relationship Between Kernel Weights and Beta-Glucan Viscosity

The relationship between average kernel weight and average beta-glucan viscosity for the Wx Lk2 N, lk2, n, and lk2 n isotypes (across genotypes) is plotted in Figure 6-5. This relationship was significant with r = -.97*. The actual data points for the four nonwaxy isotypes closely fit the regression line. The actual data points for the waxy isotypes were higher than the predicted values. The wx isotype point was outside a P = .10 prediction interval, and the wx n isotype point was outside a P = .05 prediction interval.

A similar relationship was noted for isotypes within genotypes. Within Compana (Figure 6-6) and Betzes (Figure 6-7), r = -.99**, and the data points of the nonwaxy isotypes were within the prediction interval. Most of the data points for the waxy isotypes were outside of a P = .01 prediction interval. The relationship within Titan (Figure 6-8) was not as good (r = .81), and the waxy isotype data points were not outside the prediction interval.

These results suggest that two different mechanisms were resulting in elevated beta-glucans in these isogenic barley lines:

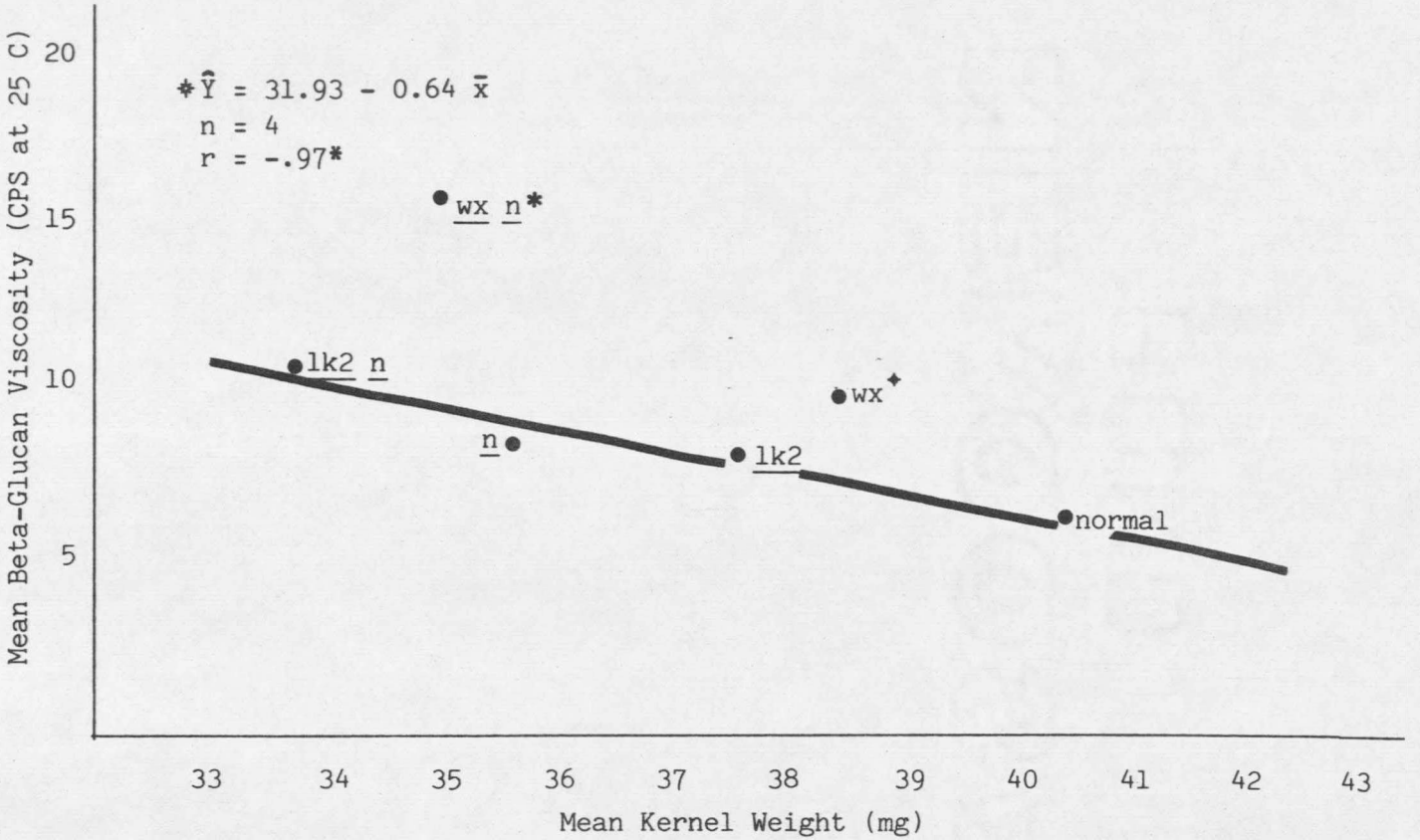

Figure 6-5. Comparison between mean kernel weight and mean beta-glucan viscosity of six isotypes across three cultivars grown in eight environments.
✦, *Significant at the 0.10 and 0.05 levels, respectively.
* Prediction line is based on a regression of mean beta-glucan viscosity vs. mean kernel weight of nonwaxy isotypes. Actual data points are plotted for all isotypes; significant differences between observed ($\bar{y}$) and predicted ($\hat{Y}$) mean beta-glucan viscosity are indicated.

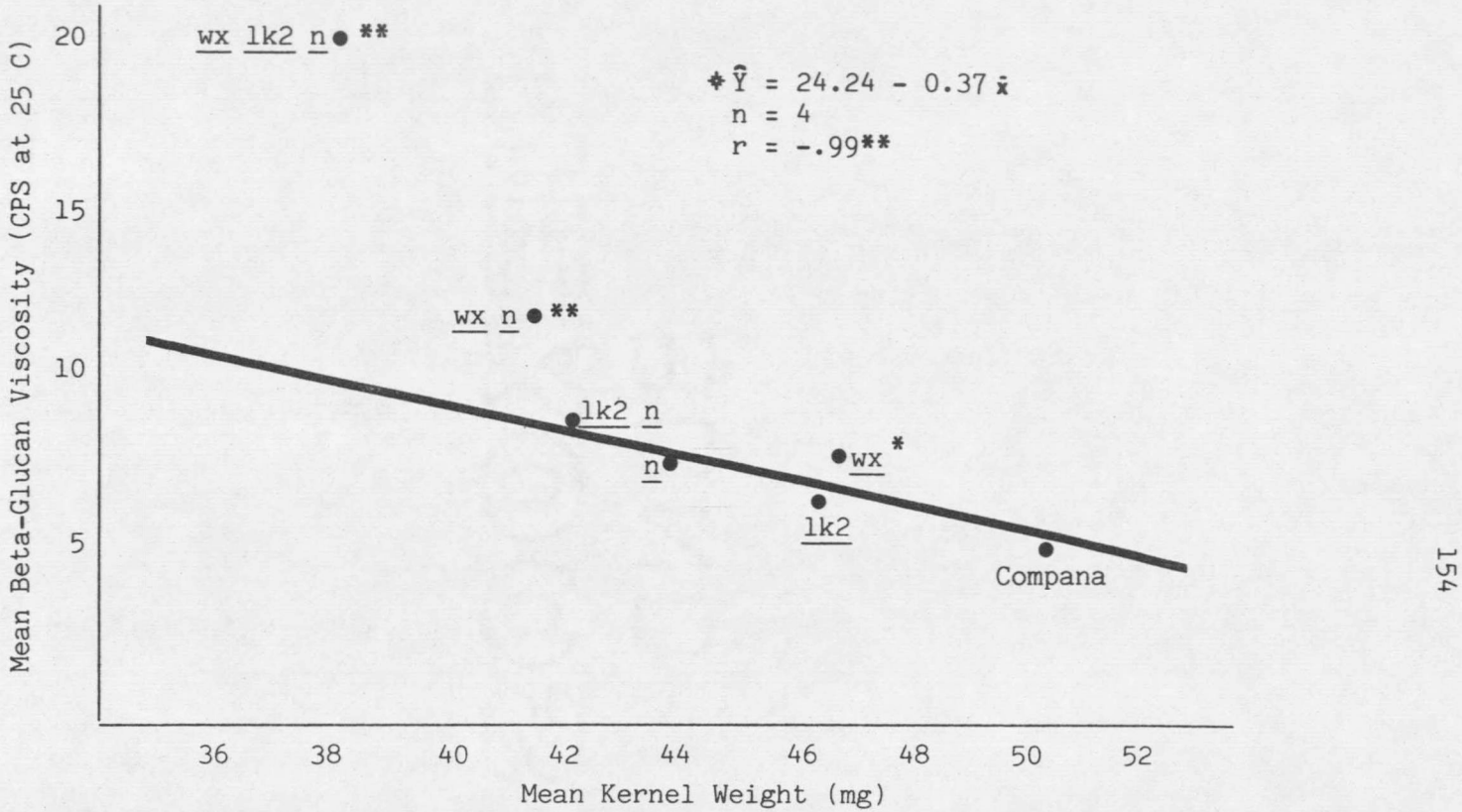

Figure 6-6. Comparison between mean kernel weight and mean beta-glucan viscosity of seven isotypes within Compana grown in eight environments.
*, **Significant at the 0.05 and 0.01 levels, respectively.
*Prediction line is based on a regression of mean beta-glucan viscosity vs. mean kernel weight of nonwaxy isotypes. Actual data points are plotted for all isotypes; significant differences between observed ($\bar{y}$) and predicted ($\hat{Y}$) mean beta-glucan viscosity are starred.

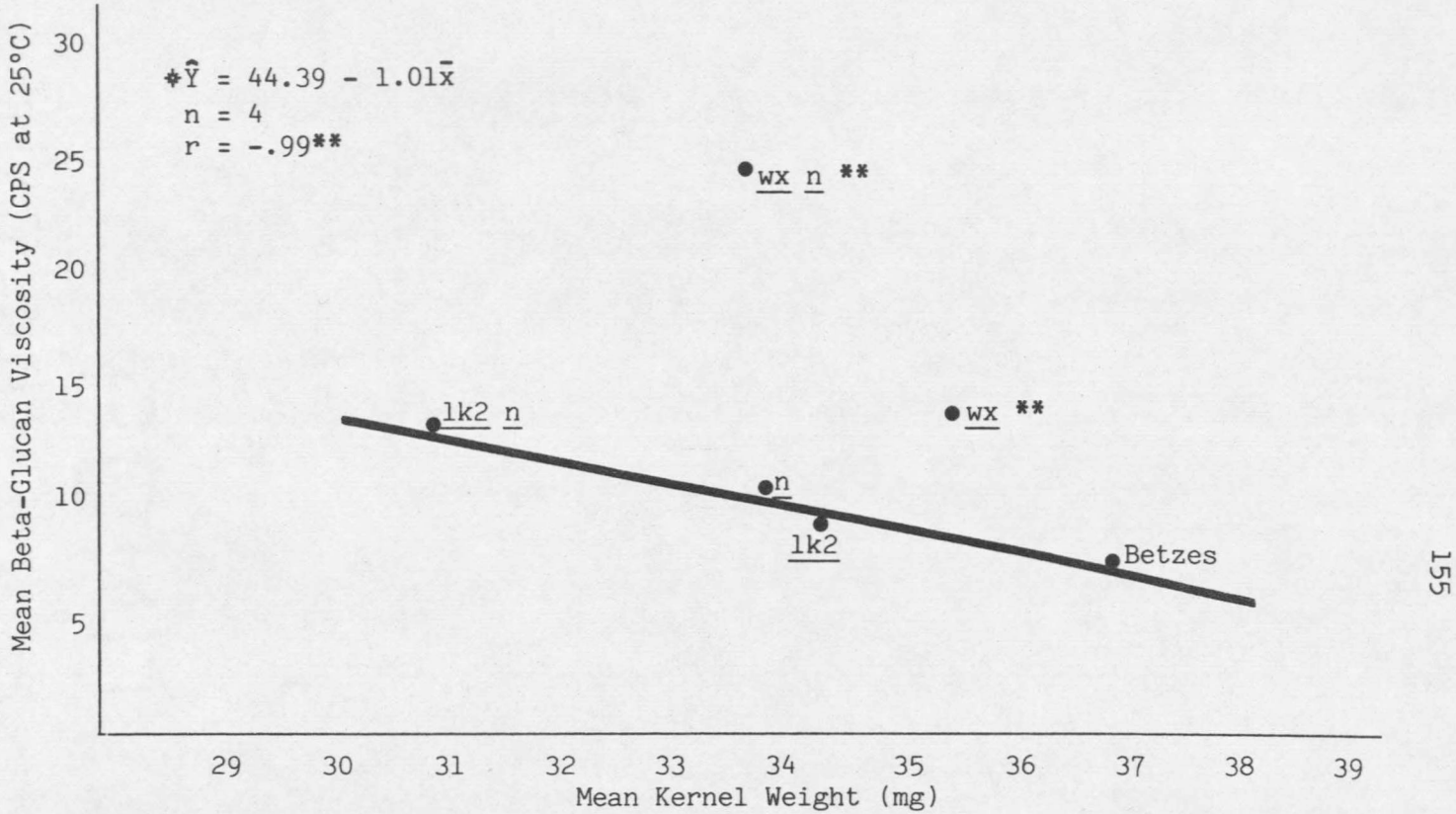

Figure 6-7. Comparison between mean kernel weight and mean beta-glucan viscosity of six isotypes within Betzes grown at eight environments.
*, **Significant at the 0.05 and 0.01 levels, respectively.
*Prediction line is based on a regression of mean beta-glucan viscosity vs. mean kernel weight of nonwaxy isotypes. Actual data points are plotted for all isotypes, significant differences between observed ($\bar{y}$) and predicted ($\hat{Y}$) mean beta-glucan viscosity are starred.

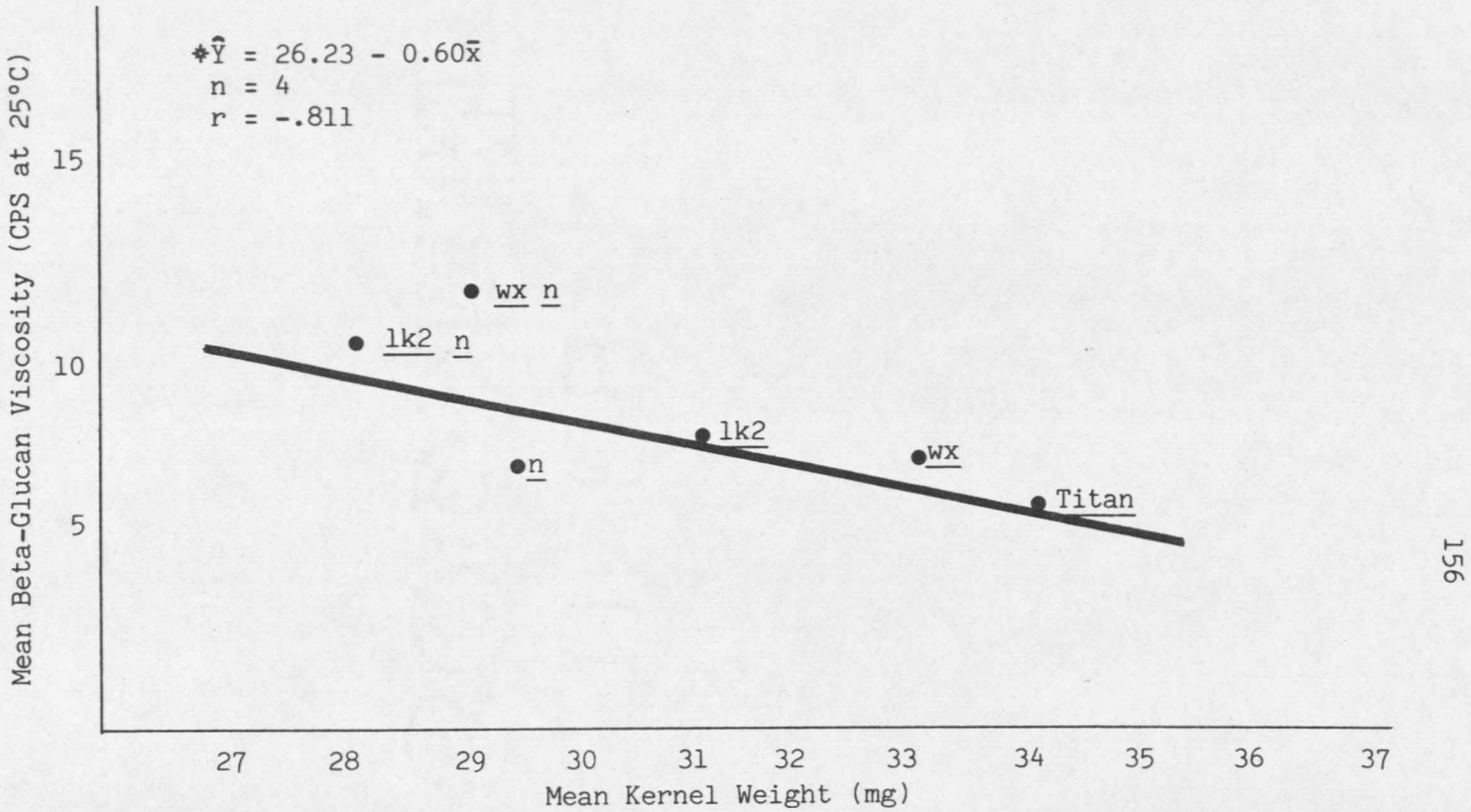

Figure 6-8. Comparison between mean kernel weight and beta-glucan viscosity of six isotypes within Titan grown in eight environments.
*Prediction line is based on a regression of mean beta-glucan viscosity vs. mean kernel weight of nonwaxy isotypes. Actual data points for both nonwaxy and waxy isotypes are plotted.

1) reduction of non-beta-glucan seed components resulting in a relative increase in beta-glucan in the lk2, n, and lk2 n isotypes; and 2) actual accumulation of beta-glucan in the wx, wx n, and wx lk2 n isotypes.

In the case of the nonwaxy isotypes, the weight of the barley seed was modified because a certain component was reduced. The hull material is removed from the seed of the hulless isotypes. In short-awn isotypes, less assimilate is reaching the seed resulting in poorly filled, low test weight kernels (McGuire and Hockett, 1981). Beta-glucans are primarily a constituent of the endosperm cell walls. Thus, when other seed components are reduced, such as crude fiber in the hulless isotypes, or starch and/or protein in the short-awn isotypes, then the relative proportion of cell walls or beta-glucans must increase.

The fact that waxy isotypes, on the whole, did not fit the beta-glucan viscosity vs. kernel weight regression line but were significantly higher, indicates that viscosities were increased in waxy isotypes beyond the concentration (cell wall-beta-glucans) effect observed for the lk2, n, and lk2 n isotypes.

The base line from which the elevated beta-glucan viscosity must be measured is the regression line. The reason that differences in beta-glucan viscosity were observed between waxy isotypes, i.e.,

wx lk2 n > wx n > wx, was because each waxy isotype started out at a different position on the regression line. For example, wx lk2 n was higher than wx n in beta-glucan viscosity because lk2 n was higher than n in viscosity; wx n was higher than wx because n was higher than the Wx Lk2 N for viscosity.

Waxy endosperm is a trait that causes a complete deletion of the amylose synthesizing capability of a seed. It is possible that with the amylose pathway deleted in waxy isotypes, starch synthesis is impeded. Sugars translocated to the seed may be diverted into other storage polysaccharides. It has been reported (Coles, 1979) that beta-glucans, under some conditions, serve as storage polysaccharides.

Percent Protein--The comparison of isotype means (across genotypes) in Figure 6-9 indicated that the wx isotype and n isotype had a higher percent protein (P = .01) than the Wx Lk2 N type. The lk2 isotype is slightly lower than the Wx Lk2 N (insignificant) in percent protein. When compared to the n isotype, the wx n isotype was significantly higher in percent protein (P = .01), and the lk2 n isotype was lower but not to a significant degree.

The comparison of genotype means indicated that Compana was significantly higher in percent protein (P = .01) than either Titan or Betzes.

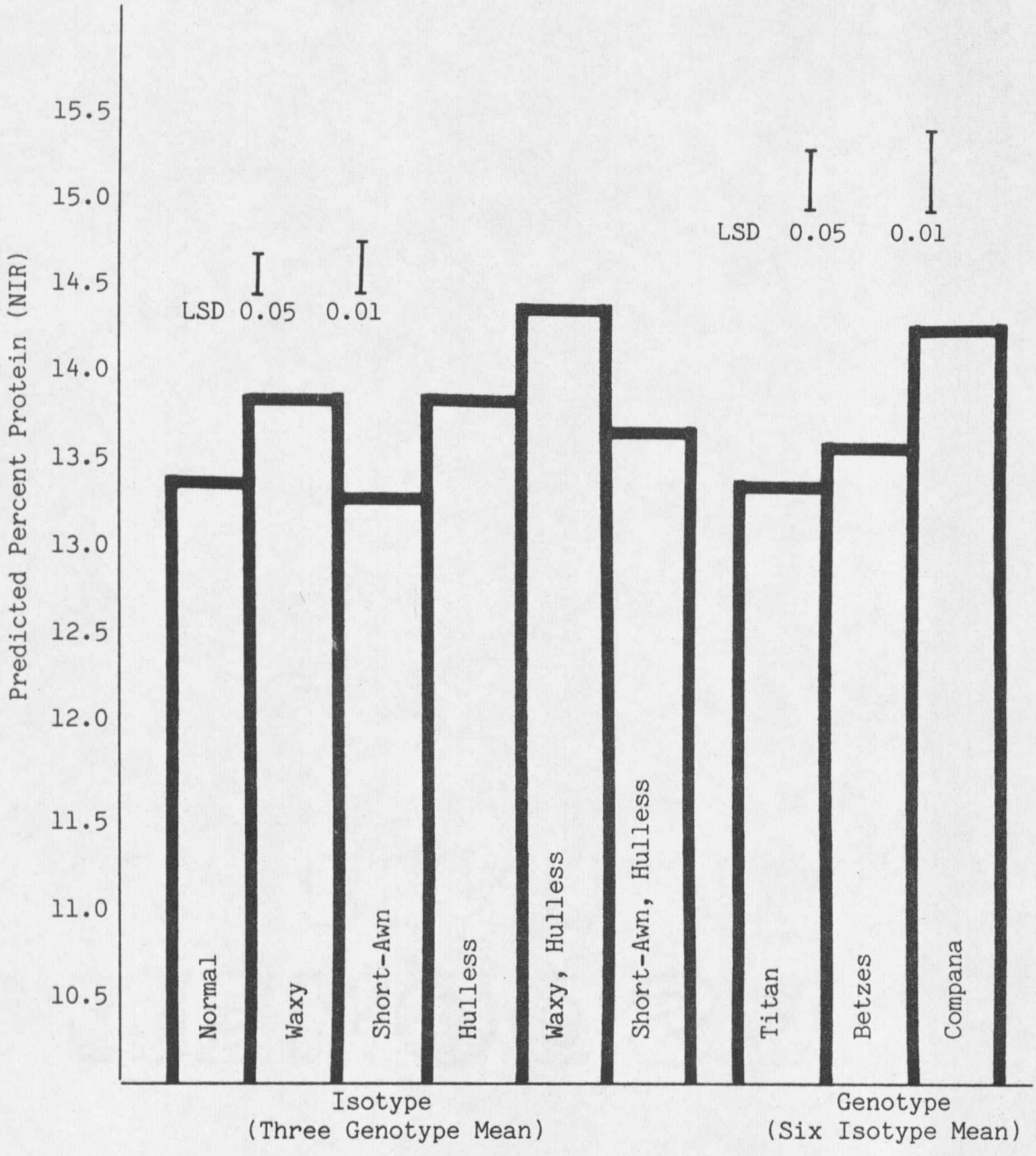

Figure 6-9. Mean predicted percent protein of six isotypes (across three genotypes) and three genotypes (across six isotypes) grown in eight environments.

The performance of isotypes within genotypes (Figure 6-10) were similar (isotype x genotype interaction nonsignificant, Table 6-2). Within Compana, the wx lk2 n isotype was not significantly different from the lk2 n or wx n isotypes.

The percent protein of the n isotypes was significantly higher than the normal types. This is because removal of the hull would dictate that percent protein content increase proportionally. However, the increase was not proportional. The kernel weight reduction of the n is 12% less than normal, while the protein increase is only 4% greater than normal. This suggests, that on a seed basis, the n isotype actually had less protein than the normal. A similar discrepancy was observed by McGuire and Hockett (1981). No explanation for this can be offered.

Significant differences in percent protein were not observed between the lk2 and normal isotypes, or the lk2 n and n isotypes. The significant reduced kernel weights of the lk2 and lk2 n isotypes would suggest that protein content increase proportionally. These results indicate that incorporation of the lk2 gene into a genotype caused a decreased protein content, on a seed basis.

The significant increase in percent protein (4%) in the waxy isotypes was proportional to the kernel weight reduction (4%). This

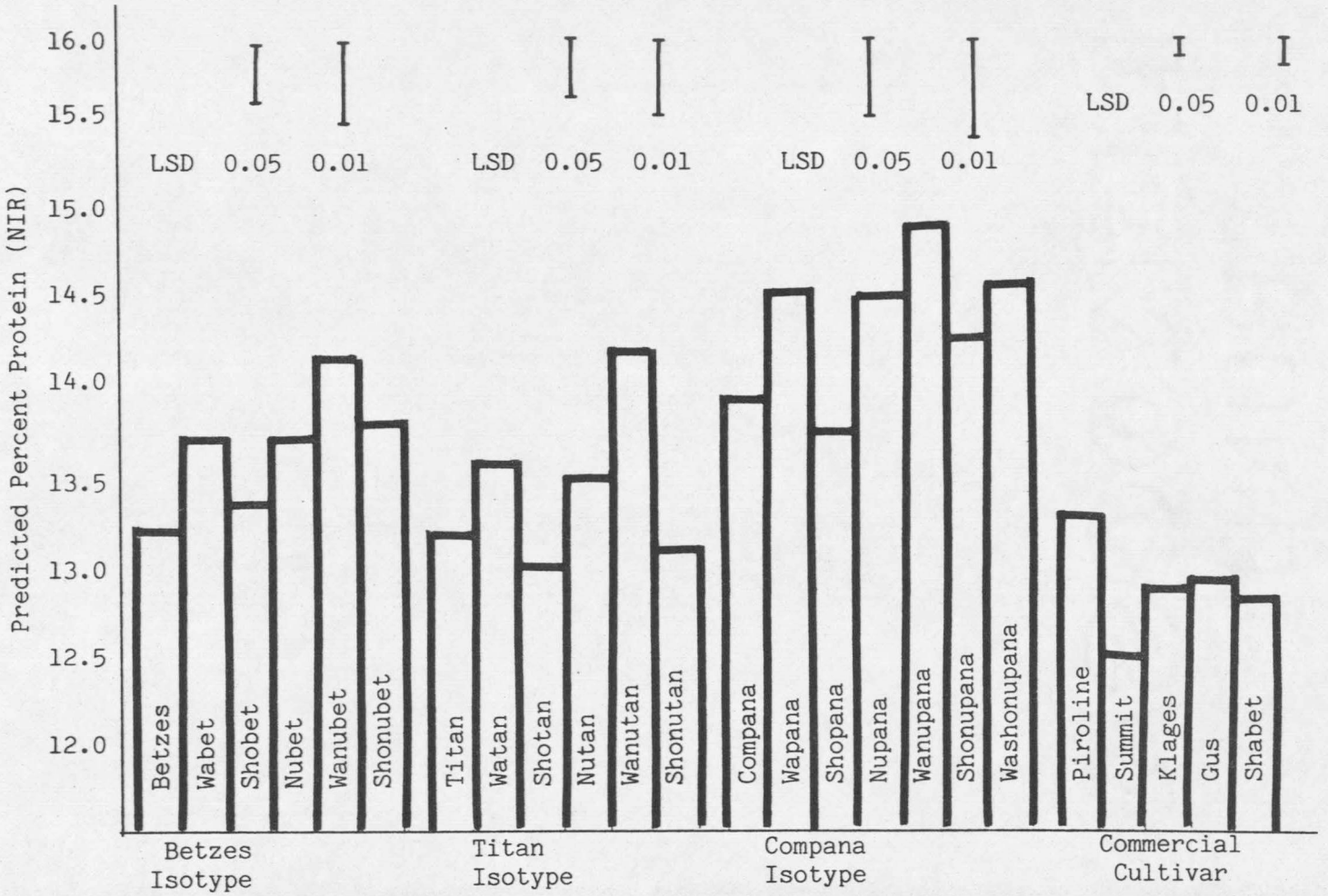

Figure 6-10. Mean percent protein for six isotypes of Betzes and Titan, seven isotypes of Compana, and five commercial cultivars grown in eight environments.

indicates that the reduction in kernel weight in the waxy isotypes was probably not due to reduced protein.

Percent Extract--The percent extract as predicted by the Technicon Infraalyzer 400 was used as a relative measure of the differences in starch content among isotypes. A high predicted percent extract should indicate high levels of starch while a low predicted percent extract should indicate lower levels of starch.

The comparison of isotype means (across genotypes) in Figure 6-11 indicated that the wx isotype was significantly lower in predicted extract than the Wx Lk2 N isotype ($P = .01$), and the wx n isotype was significantly lower than the n isotype ($P = .01$). No other differences were observed.

The comparison of genotype means (across isotypes) indicated that Betzes had higher predicted extract values than Compana ($P = .01$) and Titan ($P = .05$). Since Betzes is a find-hulled malting barley this was not surprising. Titan, also fine hulled, was significantly ($P = .01$) higher in predicted extract than Compana.

The performance of the isotypes within genotypes (Figure 6-12) was similar to their performance across genotypes (isotype x genotype interaction nonsignificant, Table 6-2). Within Compana, the wx lk2 n isotype had a significantly ($P = .01$) lower predicted extract than the lk2 n isotype.

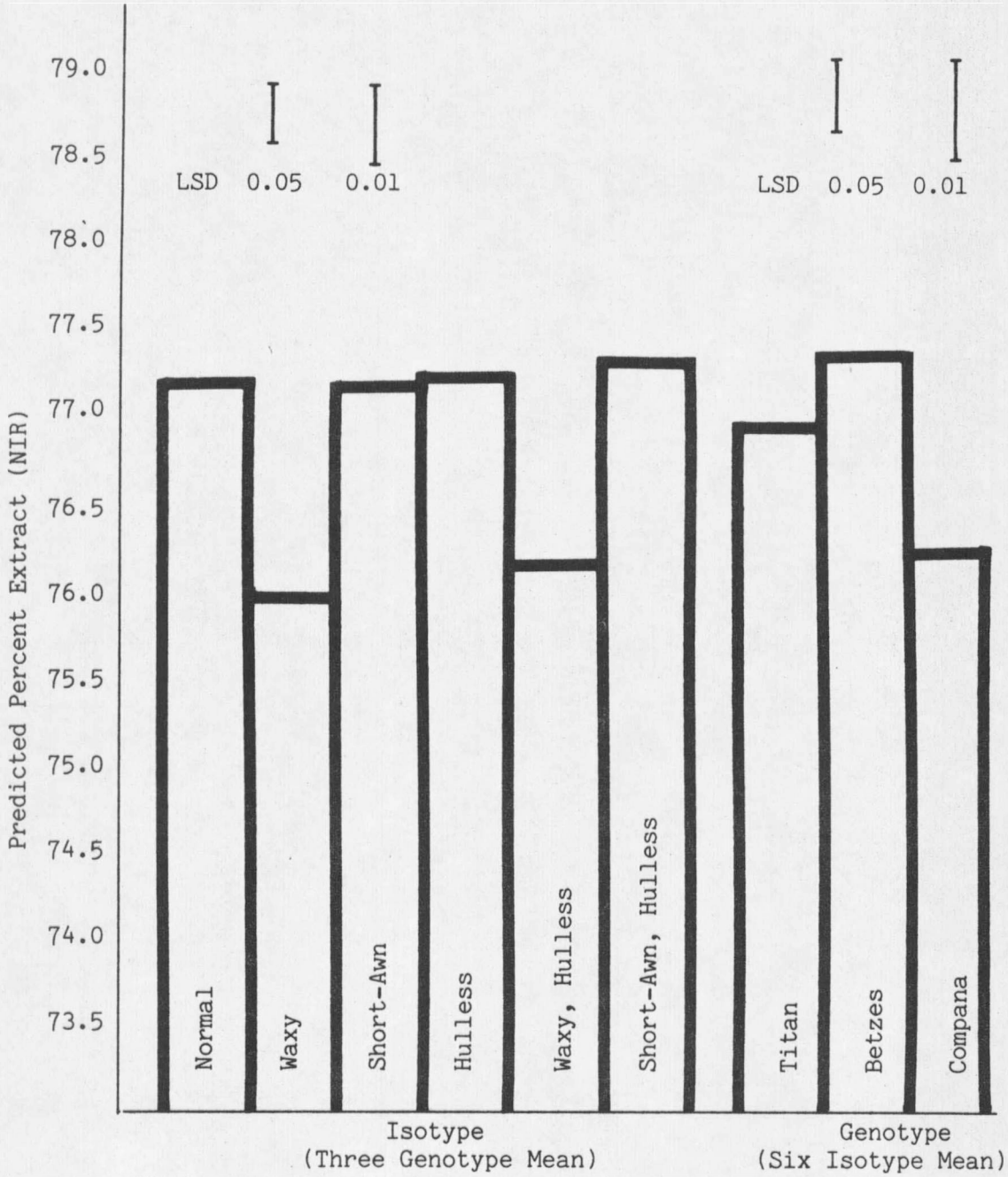

Figure 6-11. Mean predicted percent extract of six isotypes (across three genotypes) and three genotypes (across six isotypes) grown in eight environments.

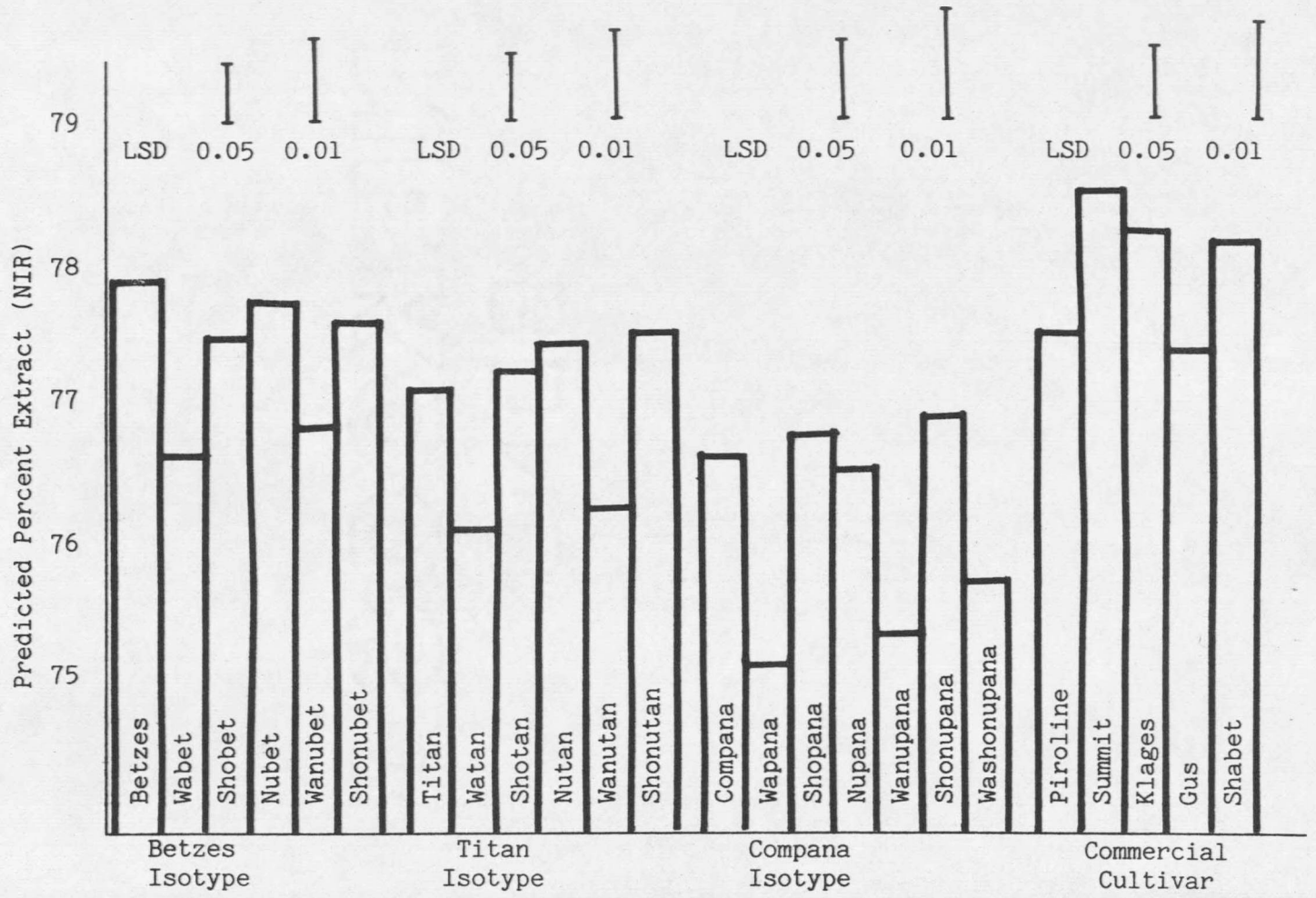

Figure 6-12. Mean percent extract of six Betzes and Titan isotypes, seven Compana isotypes, and five commercial cultivars grown in eight environments.

Without recalibrating the NIR for the isotypes used in this study, conclusions from the data should be treated with caution. However, expected differences in predicted extract between malting and feed barley were observed. In addition, the lower kernel weights of the waxy isotypes indicate a reduction in components. Since beta-glucan and protein were significantly higher, the reduction must occur elsewhere.

Alpha-Amylase Activity--The comparison of isotype means (across genotypes) in Figure 6-13 indicated that there were no isotypes with unusual amounts of alpha-amylase. Ripe, sound seed will have enzyme activity ranging from 10-100 CBA dye release units. None of the isotypes exceeded 30 dye release units. However, significant differences did exist among isotypes with respect to these small amounts of enzyme. None of the differences between the Wx Lk2 N and the wx, lk2, or n isotypes were significant. The difference between the n isotype and the wx n was also not significant. The lk2 n isotype was significantly (P = .05) lower in alpha-amylase activity than the n isotype. A comparison between the highest activity isotype wx n, and the lowest activity isotype, lk2 n, was also significant (P = .01).

The comparison of genotype means (across isotypes) indicated that Titan had significantly (P = .05) higher levels of alpha-amylase than

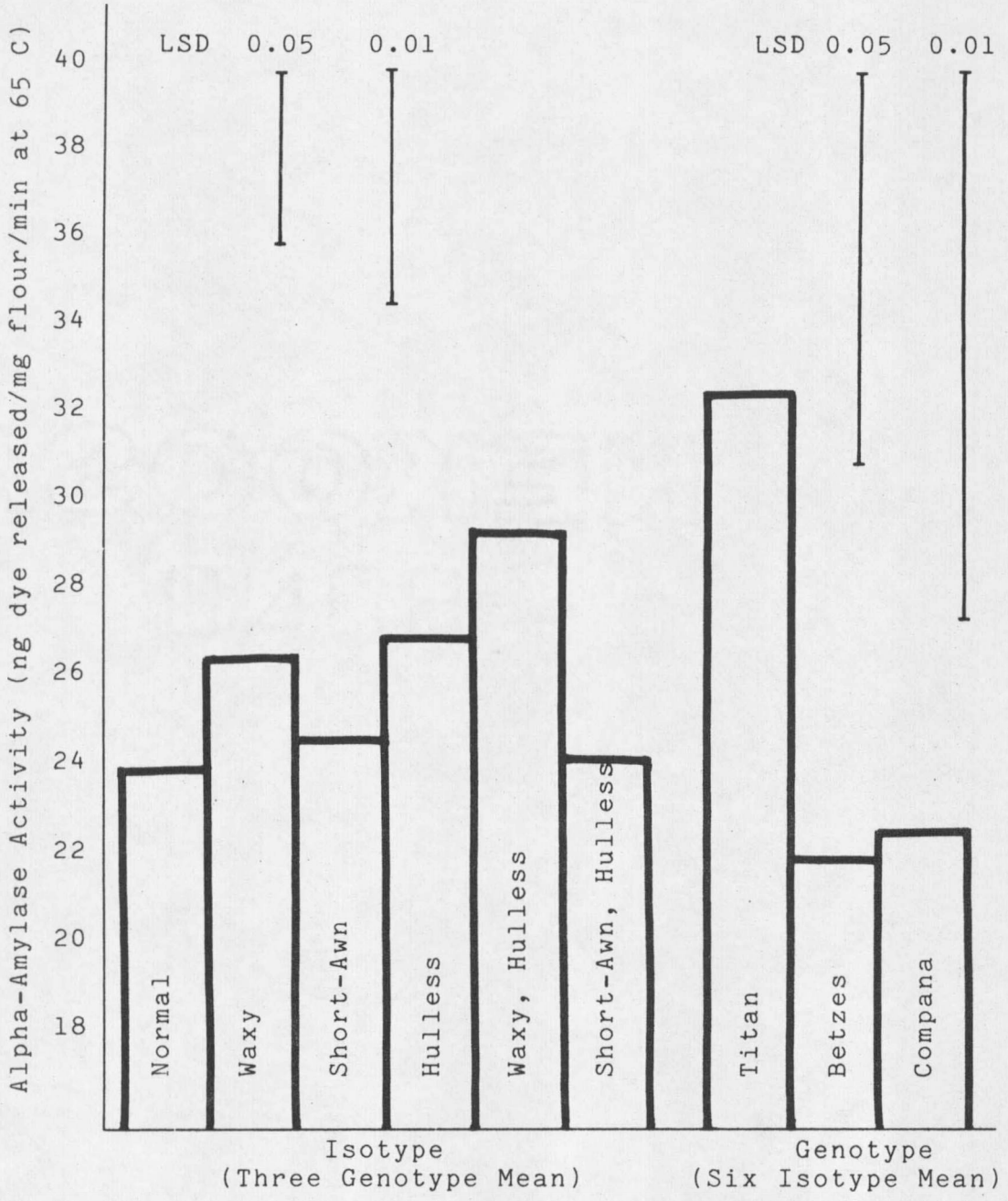

Figure 6-13. Mean alpha-amylase activity in nongerminated samples of six isotypes (across three genotypes and three genotypes (across six isotypes) grown in seven environments.

Betzes and Compana. This was observed in a comparison of 50 cultivars which included Titan, Betzes, and Compana in Chapter 4.

The behavior of the isotypes within genotypes was not consistent (Figure 6-14), but a significant isotype x genotype interaction was not detected (Table 6-2). Within Compana, the wx lk2 n isotype was significantly (P = .05) lower in alpha-amylase activity than the wx n isotype, and similar to the lk2 n isotype.

Few generalizations could be made concerning the small amounts of alpha-amylase found in sound barley seed. Small insignificant increases in alpha-amylase was observed in the same isotypes which had significant increases in percent protein. The alpha-amylase increases may not have been significant since alpha-amylase is only one of the many proteins found in a barley seed. The decrease in alpha-amylase activity in the lk2 n isotype may be related to the lack of an increase in protein in this isotype.

The relationship between protein and alpha-amylase was tested by regressing the mean isotype alpha-amylase activity vs. the mean isotype percent protein (across varieties). Figure 6-15 shows this relationship was significant (r = .82*).

The same regression was plotted for isotypes within the three varieties (Figure 6-16). A strong relationship between protein and alpha-amylase activity was observed in Titan isotypes (r = .95**)

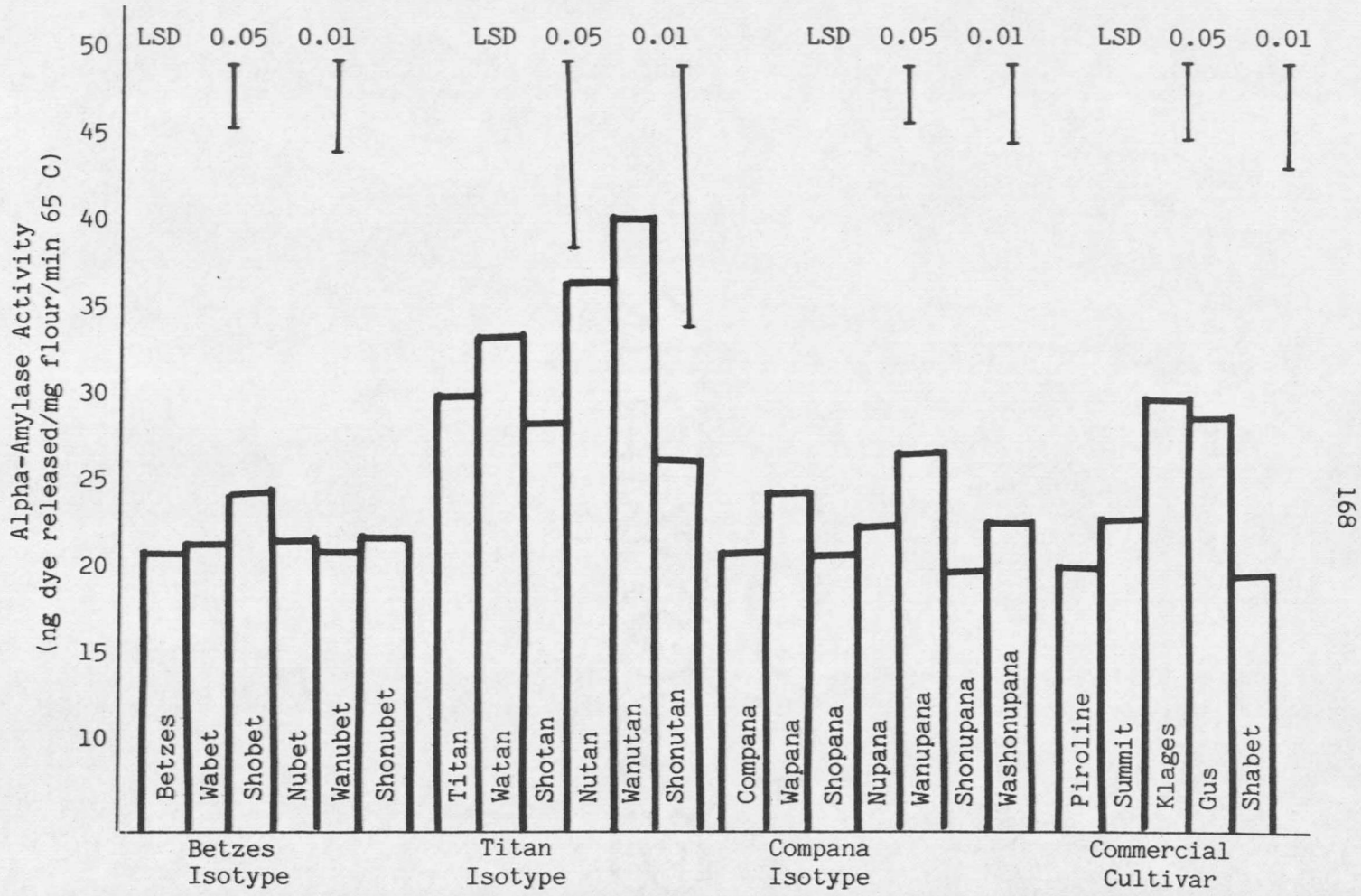

Figure 6-14. Mean alpha-amylase activity of six isotypes of Betzes and Titan, seven isotypes of Compana and five commercial cultivars grown in seven environments.

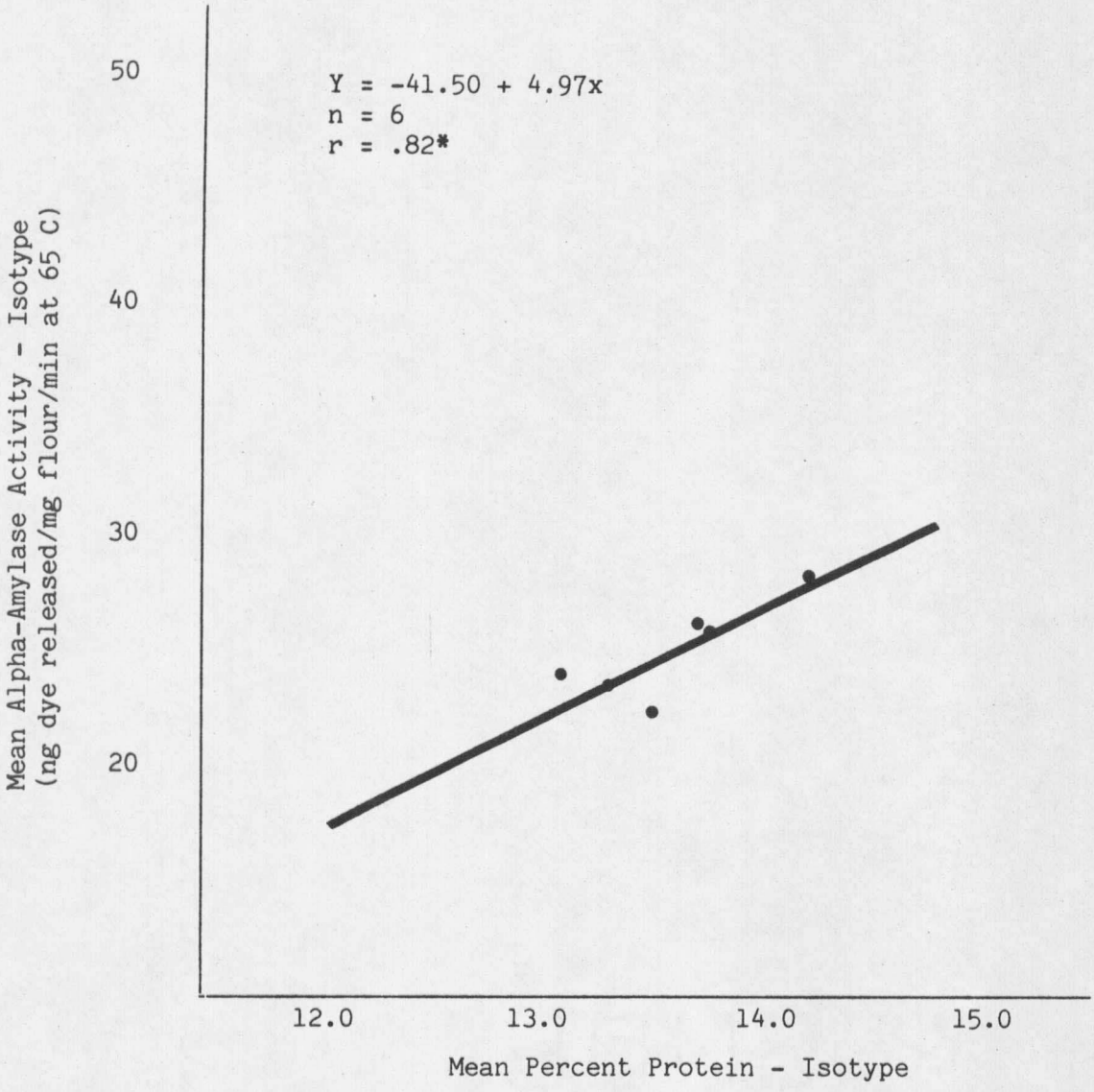

Figure 6-15. Comparison between mean alpha-amylase activity and mean percent protein of six isotypes across three genotypes grown in seven environments.
*Significant at the 0.05 level.

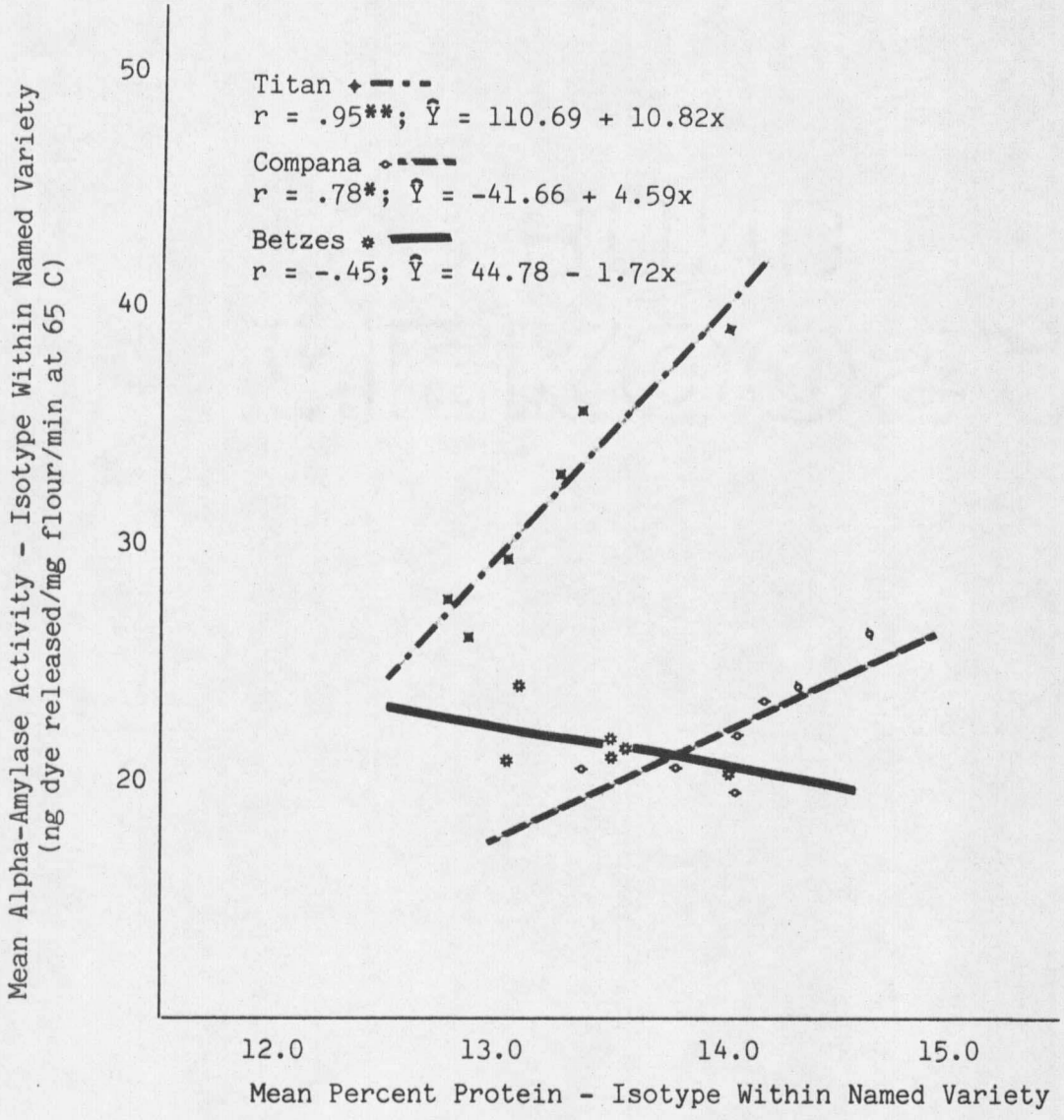

Figure 6-16. Comparison between mean alpha-amylase activity and mean percent protein of six isotypes within Betzes and Titan and of seven isotypes within Compana grown in seven environments.
*, **Significant at the 0.05 and 0.01 levels, respectively.

and Compana isotypes (r = .78*). This relationship was not observed within the Betzes isotypes (r = -.45). Significant differences were detected among the regression coefficients (b_{yx}). The positive regression coefficients of Titan (b_{yx} = 10.82) and Compana (b_{yx} = 4.59) were significantly (P = .01 and .05, respectively) different from the negative regression coefficient of Betzes (b_{yx} = -1.72). The larger regression coefficient of Titan was significantly (P = .05) different from that of Compana. These results indicate that the observed positive correlation between mean isotype percent protein and mean isotype alpha-amylase activity was significantly influenced by background genotype.

Summary

The incorporation of the waxy, short-awn, and hulless genes into barley genotypes resulted in significant reductions in kernel weight for all isotypes. The significant kernel weight differences observed across genotypes were as follows: normal > waxy > short-awn > hulless > waxy, hulless > short-awn, hulless.

Beta-glucan viscosity was inversely related to kernel weight for all nonwaxy isotypes. Waxy isotypes had viscosity levels significantly above the level predicted from the kernel weight reduction. This in-

dicates that waxy isotypes are increasing in viscosity beyond a simple beta-glucan concentration effect based upon reduced kernel weight.

As an indirect measure of starch the percent extract was predicted using near infrared reflectance analysis. All waxy isotypes were significantly lower in predicted extract than the nonwaxy counterparts. No other significant differences were observed for isotypes.

Percent grain protein was significantly higher in hulless isotypes when compared to normal, and in waxy isotypes when compared to their nonwaxy counterparts. The short-awn showed a tendency to reduce percent protein in hulless isotypes (not significant).

No isotype produced high levels of alpha-amylase. Significant differences in the small amounts of alpha-amylase found in sound seed were observed. Waxy, hulless isotypes contained significantly more enzyme activity than short-awn, hulless isotypes. These isotypes also represented the extremes for protein content, with waxy, hulless the highest and short-awn, hulless the lowest. The decrease in alpha-amylase activity which resulted upon adding the short-awn gene to the hulless isotype suggests this trait may not be desirable in cultivars grown for use by industries requiring alpha-amylase activity in nongerminated seed.

Differences among the three genotypes were observed for all response variables. The effect of background genotype was generally not to alter the relative effect of certain genes but to amplify or dampen their impact. In the case of beta-glucan viscosity the inclusion of the waxy gene into all three genotypes caused an increase. However, the increase was of a much higher magnitude in Betzes than in the Compana or Titan backgrounds. This genotype x isotype interaction is important to the plant breeder, allowing him to produce cultivars with or without high viscosity levels, depending upon the requirements of the industry.

CHAPTER 7

THE EFFECT OF ENVIRONMENT AND ISOTYPE UPON SEED QUALITY CHARACTERISTICS IN THREE CULTIVARS

Introduction

The significant interactions observed in the combined split-plot analysis and the individual isotype within variety analyses for the response variables kernel weight, beta-glucan viscosity, predicted percent extract, and alpha-amylase activity were examined by regression analysis. No significant interactions were observed for percent protein in the combined analysis.

Using regression analysis, a researcher can observe the performance of genotypes (cultivars) across environments (G x E), isotypes across environments (I x E), and genotypes across isotypes (G x I) as regression lines. Visual examination of the regression lines and the associated $\bar{y}$, r^2, and b_{yx} values, provides the researcher insight into the associated source of the G x E, I x E, and G x I interactions.

The G x I interaction is especially important to the plant breeder since the very essence of plant breeding is utilizing favorable G x I interactions in new improved cultivars. This quantity has seldom been

measured by plant breeders but, none the less, is appreciated. By using isogenics within a number of genetic backgrounds, as has been done in this study, this very important interaction can be measured.

Materials and Methods

The experimental material used in this study is described in Chapter 6. The significance levels for the interactions discussed in this chapter were obtained from Tables 6-2 and 6-3 in Chapter 6.

The regression analysis for the genotype x environment (G x E) interaction was done by regressing the mean response of each genotype (all isotypes) in each environment against the environment mean. This produced three regression lines, one for each cultivar. A unity line was plotted by regressing the environment mean against itself. The regression coefficient (b_{yx}) and coefficient of determination (r^2) were calculated for each regression line.

The regression analysis for the genotype x isotype (G x I) interaction was done by regressing the mean response of each isotype within a genotype (wx, lk2, n, wx n, lk2 n) against the mean response of that isotype (all genotypes, all environments). This produced three regression lines, one for each genotype. A unity line was plotted by regressing the isotype mean against itself. The regression coefficient and coefficient of determination were calculated for each regression line.

The regression analysis for the isotype x environment (I x E) interaction was done by regressing the mean of each mutant isotype (all genotypes) in each environment against the mean of the normal isotype (all genotypes) in that environment. This produced five regression lines, one for each mutant isotype. The normal type was regressed against itself to produce a unity line. The regression coefficient and coefficient of determination were calculated for each regression line.

The regression analysis for the I x E interaction within each of the three genotypes was done by regressing the mean response of each mutant within a genotype in each environment against the mean of the normal type of that genotype in that environment. The normal type was regressed against itself to produce a unity line. This analysis was similar to the procedure described in the previous paragraph except it was performed for each cultivar separately.

For all of the described regressions, the assumption was made that the x-coordinate represents a range of environments (genetic or physical) and that the environments run from low to high beginning at the x, y axis and moving to the positive (right hand) side. Thus, the x-variable was not fixed. A second assumption was made that for each x, a sample value of y is drawn from a normal population with a

mean $u = a + B x$ and a constant variance $_{yx}^{2}$ (Snedecor and Cochran, 1967). However, the above assumptions are not completely justified since the independent variable (x) was not measured without error.

The three factor interaction, isotype x genotype x environment, cannot be simply analyzed in a two-dimensional regression. For this complex interaction, the separate isotype x environment regression analyses for each genotype (Betzes, Titan, and Compana) were compared. In this manner a qualitative estimate of the sources of the triple interaction was obtained.

Results and Discussion

Interpretation of Regression Analysis

The associated variance of the two-factor interactions discussed in this chapter can be broken down into the two components of a regression analysis: sums of squares due to regression (SS regression), and sums of squares due to deviations from regression (SS deviation). Thus, the regression lines calculated for each of the two-factor interactions (G x E, G x I, and I x E) have an associated SS regression and SS deviation. If these values for each regression line are summed, and the SS environment (or, SS about the independent variable, x) subtracted, the resulting difference is equal to the sums of squares due to the interaction (SS interaction) (Finlay and Wilkinson, 1963).

For the interactions in which the mean values for environment or isotype were used as the x-array (G x E and G x I), the SS regression for the unity line was not used in the above calculation. In the cases where the mean value of the normal isotype was used as the x-coordinate (I x E), the SS regression for the unity line had to be included in the calculation in order to obtain the relationship: SS Regression + SS Deviation - SS environment = SS interaction.

The coefficient of determination (r^2) for each regression line is a measure of the percent of interactional variability that can be accounted for by regression. Conversely, the quantity, $1 - r^2$, is a measure of the deviation from regression and, thus, variability unaccounted for by the regression equation (Snedecor and Cochran, 1967).

The slope of the regression line (b_{yx}) is a measure of how a particular isotype or genotype responds to changing environments when compared to a normal type, or average genotype. A t-test was used to test whether the b_{yx} for a particular isotype or genotype was different from the unity line ($b_{yx} = 1$) (Ullrich, 1978).

The variation that appears in the analysis of variance for two-factor interactions is represented by 1) the deviations of the b_{yx} values from a slope of one, and 2) by the amount of unassociated variation measured by the quantity, $1 - r^2$ (Ullrich, 1978). These

two factors were used to evaluate each two-factor interaction in this chapter.

If the r^2 values were significant for a set of b_{yx} values assigned to a specific interaction, then the evaluation of the possible sources of interaction was based on examination of the b_{yx} values. If some nonsignificant r^2 were observed in a set then the unassociated variation had to be considered. If no significant r^2 values were observed for a set of b_{yx} values for a particular interaction, the evaluation of b_{yx} values was considered inappropriate since the regression does not account for a significant portion of the variability associated with the interaction.

The principle test of b_{yx} values was against a slope of one. However, with the regression model, the cumulative effect of all differences of the b_{yx} from one could account for the interaction. Thus, cases did arise where no single b_{yx} value was significantly different from one but the sum of all differences from 1 was significant. A test to detect differences among b_{yx} values (Snedecor and Cochran, 1967) was performed in these situations.

The three-factor interaction represents the amount of variation remaining after all the main effects and two-factor interaction variation has been removed. Three-factor interactions are difficult

to visualize as well as analyze. An attempt was made to at least perform a qualitative analysis of the three-factor interaction (when significant) by inspecting the I x E regression analysis for the individual genotypes. The occurrence of large amounts of unassociated variation ($1 - r^2$) for the I x E interaction within a specific genotype would represent a possible source of the three-factor interaction (response only explainable by specific genotype x isotype x environment combination).

Kernel Weight

In the combined analysis of variance (Table 6-2), the G x E, G x I, and I x E interactions were significant for kernel weight.

The r^2 values for the G x E interaction (Figure 7-1) were significant for all three genotypes. However, quite a bit of unassociated variation was observed for Betzes ($1 - .56$) with a b_{yx} very near 1.0. The b_{yx} values for Compana ($b_{yx} = 1.66^{**}$) and Titan ($b_{yx} = 0.55^{**}$) were significantly different from one. This differential response of the three genotypes to increasing kernel weight environments was the probable cause of the interaction.

The r^2 values for the G x I interaction (Figure 7-2) were significant for all three genotypes. While no significant differences were observed for b_{yx} values when compared to the unity line, there were

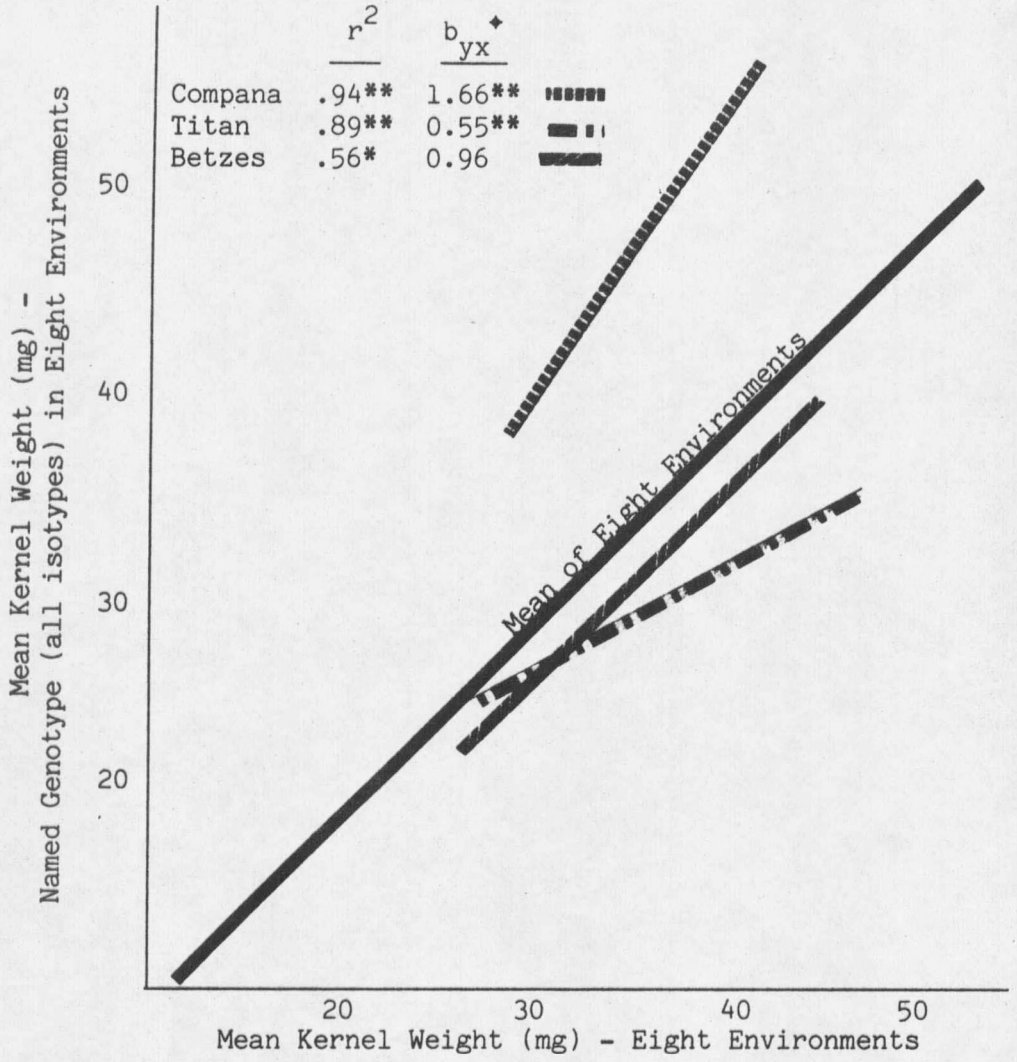

Figure 7-1. Kernel weight relationship between the means of Titan, Betzes, and Compana (all isotypes) and the mean of all entries grown in eight environments.
*,**Significant at the 0.05 and 0.01 levels, respectively.
✦ Significant differences from a slope of 1.0 are starred.

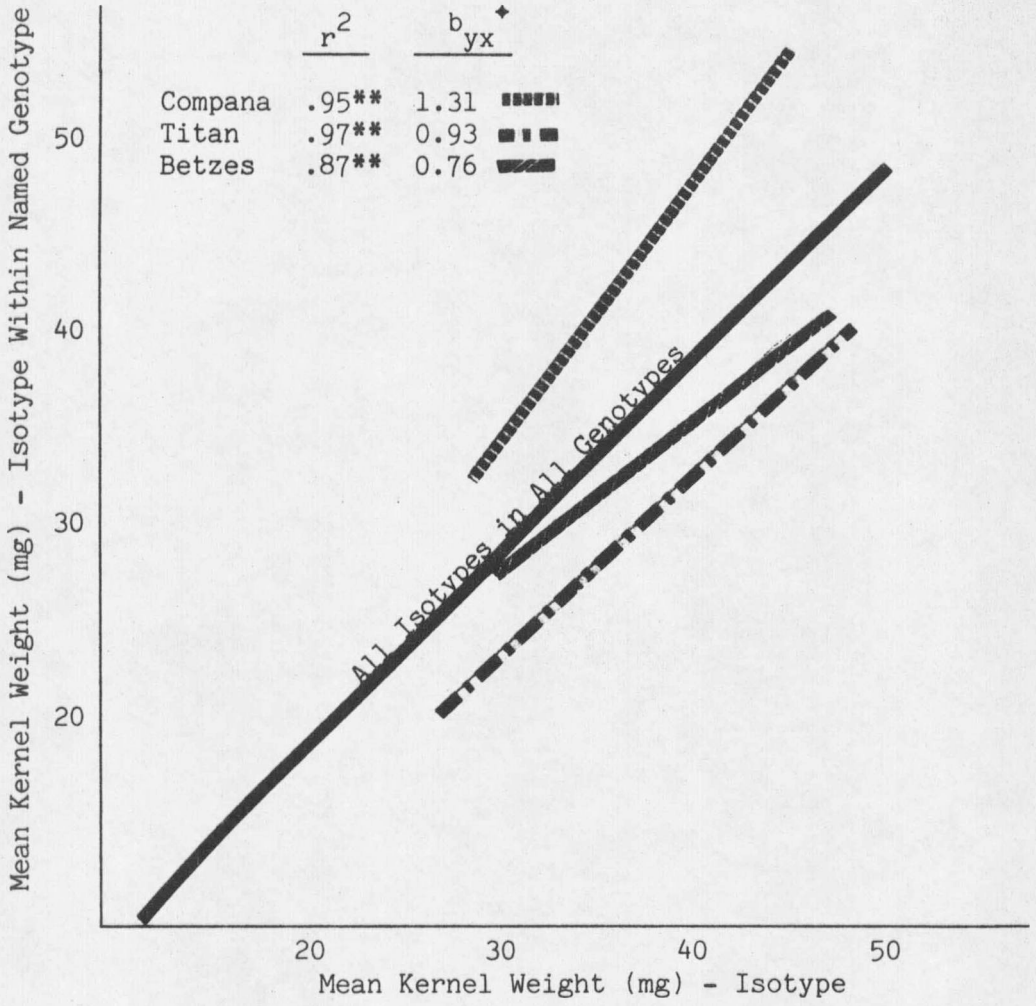

Figure 7-2. Kernel weight relationship between the means of six isotypes of Titan, Betzes, and Compana (all environments) and the mean of the six isotypes in all genotypes (all environments).
**Significant at the 0.01 level.
✦ Significant differences from a slope of 1.0 are starred.

differences between the extreme b_{yx} values. Compana (b_{yx} = 1.31) had a significantly higher (P = .05) b_{yx} than Betzes (b_{yx} = 0.76). Thus, the cumulative effect of all the b_{yx} deviations from a slope of one could account for the observed interaction indicating differences in magnitude of response between genotypes.

These kernel weight regression analyses indicate that Compana was more strongly affected by increasing kernel weight environments or isotypes than Titan or Betzes. Titan was the least affected of the three varieties when examined across environments, and Betzes was the least affected when examined across kernel weight reducing isotypes.

The r^2 values for the I x E interaction (Figure 7-3) were highly significant for all five isotypes. Very little unassociated variation was observed. The b_{yx} values for all isotypes except lk2 were significantly different from the normal isotype (b_{yx} = 1.0). Clearly, in this case, the interaction was due to the lesser response of the isotypes to increasing kernel weight environments.

In the isotype within genotype analysis, significant I x E interactions were observed within Compana and Betzes (Table 6-3). Since the G x I x E interaction was nonsignificant in the combined analysis (Table 6-2), trends similar to those found in the combined I x E analysis were observed. Within Compana (Figure 7-4), the b_{yx} value

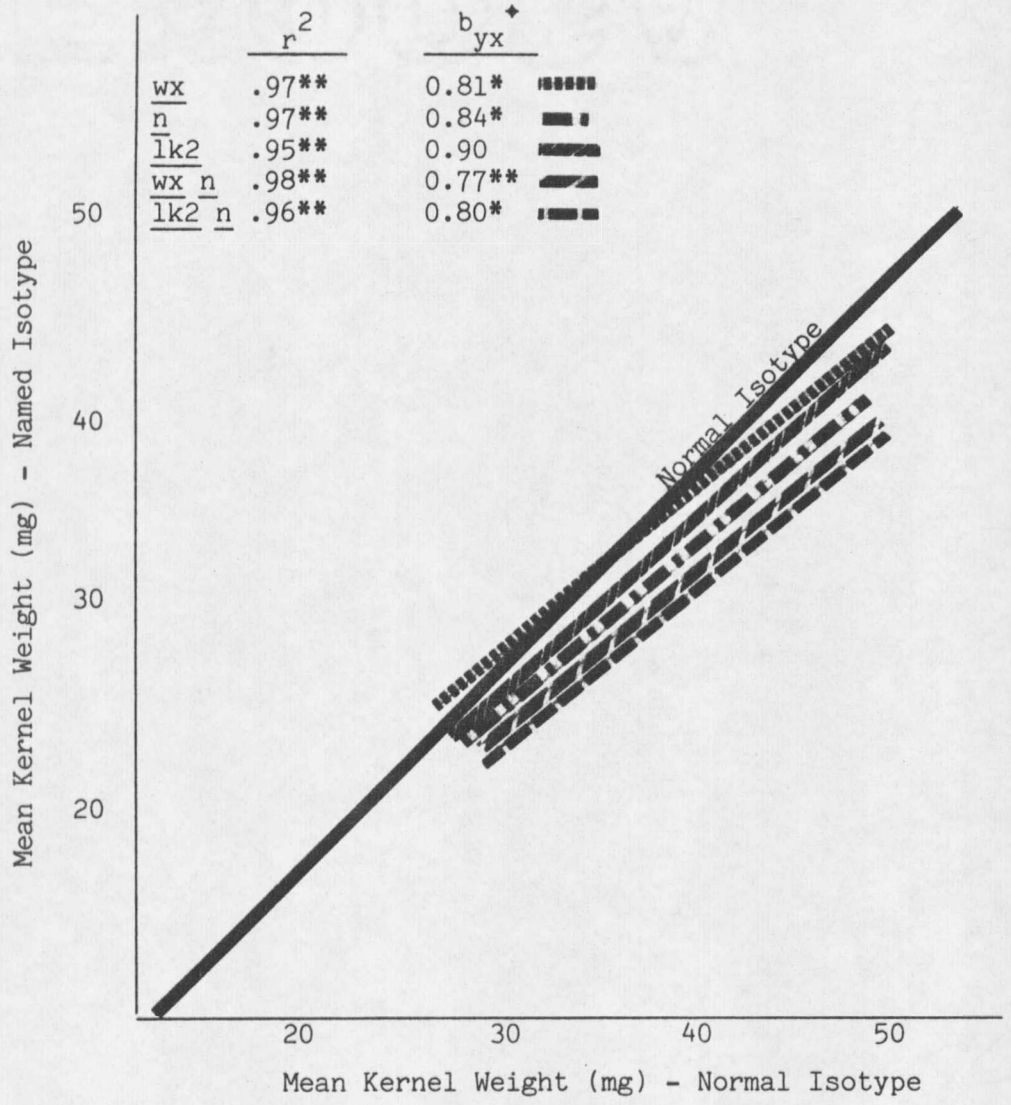

Figure 7-3. Kernel weight relationship between the means of five mutant isotypes (all genotypes) and the mean of the normal isotype (all genotypes) grown in eight environments.
*, **Significant at the 0.05 and 0.01 levels, respectively.
✦Significant differences from a slope of 1.0 are starred.

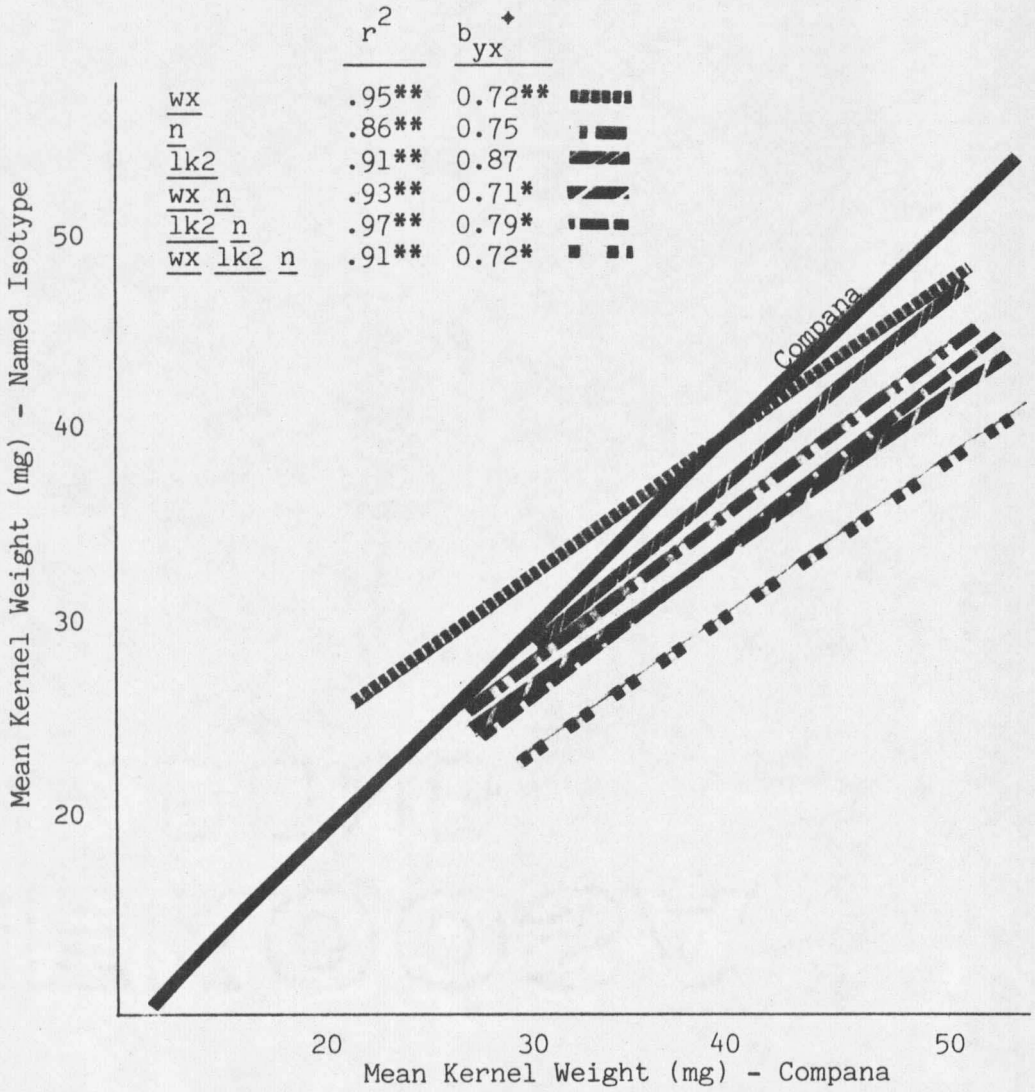

Figure 7-4. Kernel weight relationship between the means of six mutant isotypes of Compana and the mean of Compana grown in eight environments.
*, **Significant at the 0.05 and 0.01 levels, respectively.
✦Significant differences from a slope of 1.0 are starred.

of the wx lk2 n isotype (0.79*) was significantly lower than the normal isotype. These results indicated that, with the exception of the lk2 isotype, mutant isotypes not only produced seeds of lower kernel weight, but were less able to utilize environments that favored grain filling.

Beta-Glucan Viscosity

The combined analysis of variance (Table 6-2) showed that the G x E, G x I, I x E, and G x I x E interactions were significant for beta-glucan viscosity.

The r^2 values for the G x E interaction (Figure 7-5) were significant for all three genotypes. The b_{yx} values for Compana (0.52**) and Betzes (1.67**) were significantly different from one. The differential magnitude of the response of the three genotypes to environment resulting in increased beta-glucan viscosity was the cause of this interaction.

The r^2 values for the G x I interaction (Figure 7-6) were significant for all three genotypes. The b_{yx} values for Compana (0.66**), Titan (0.58*), and Betzes (1.77*) were significantly different from one, and obviously, Compana and Titan were different from Betzes. Quite clearly the interaction was the result of the differential

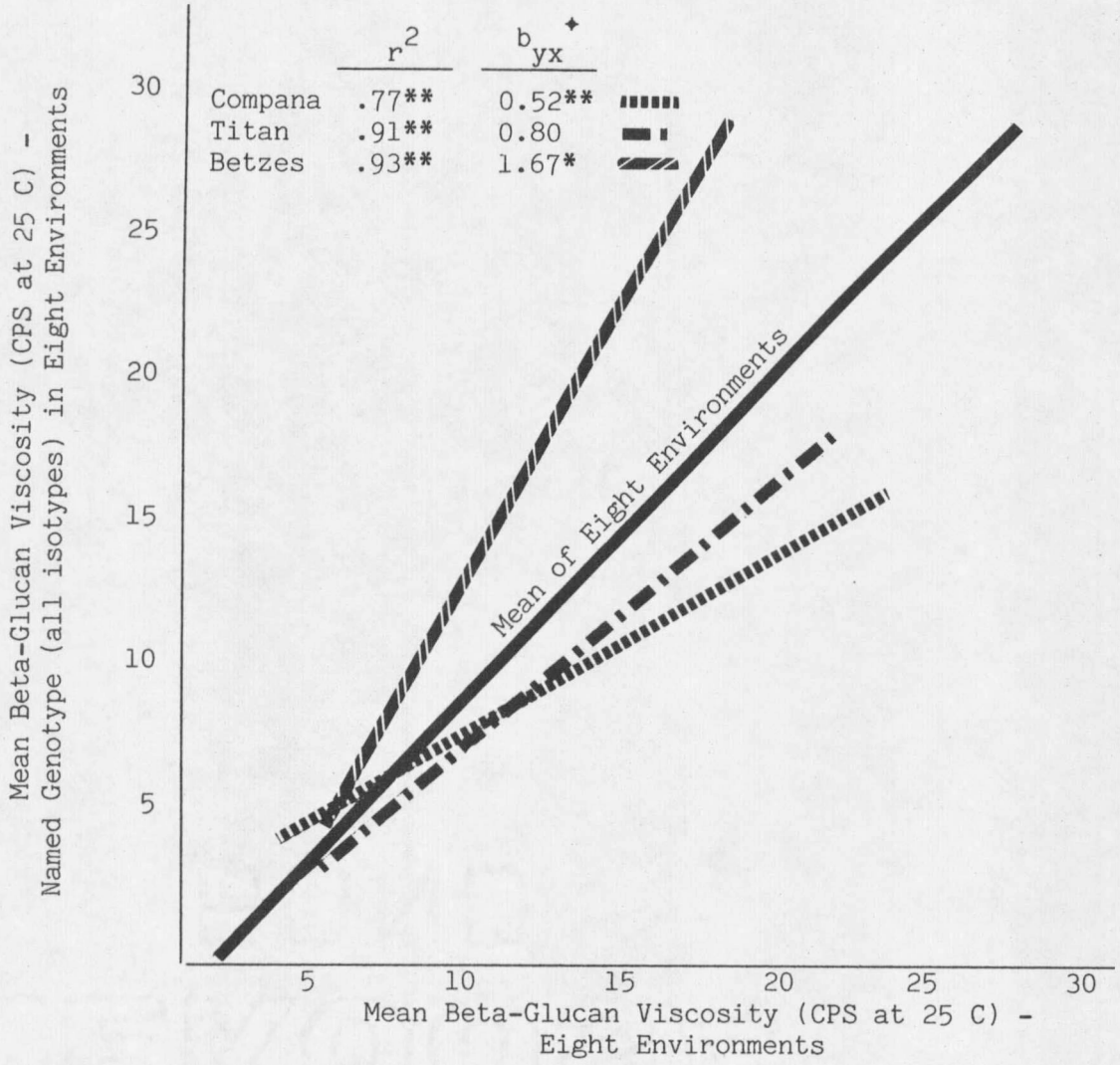

Figure 7-5. Beta-glucan viscosity relationship between the means of Titan, Betzes, and Compana (all isotypes) and the mean of all entries grown in eight environments.
*, **Significant at the 0.05 and 0.01 levels, respectively.
✦Significant differences from a slope of 1.0 are starred.

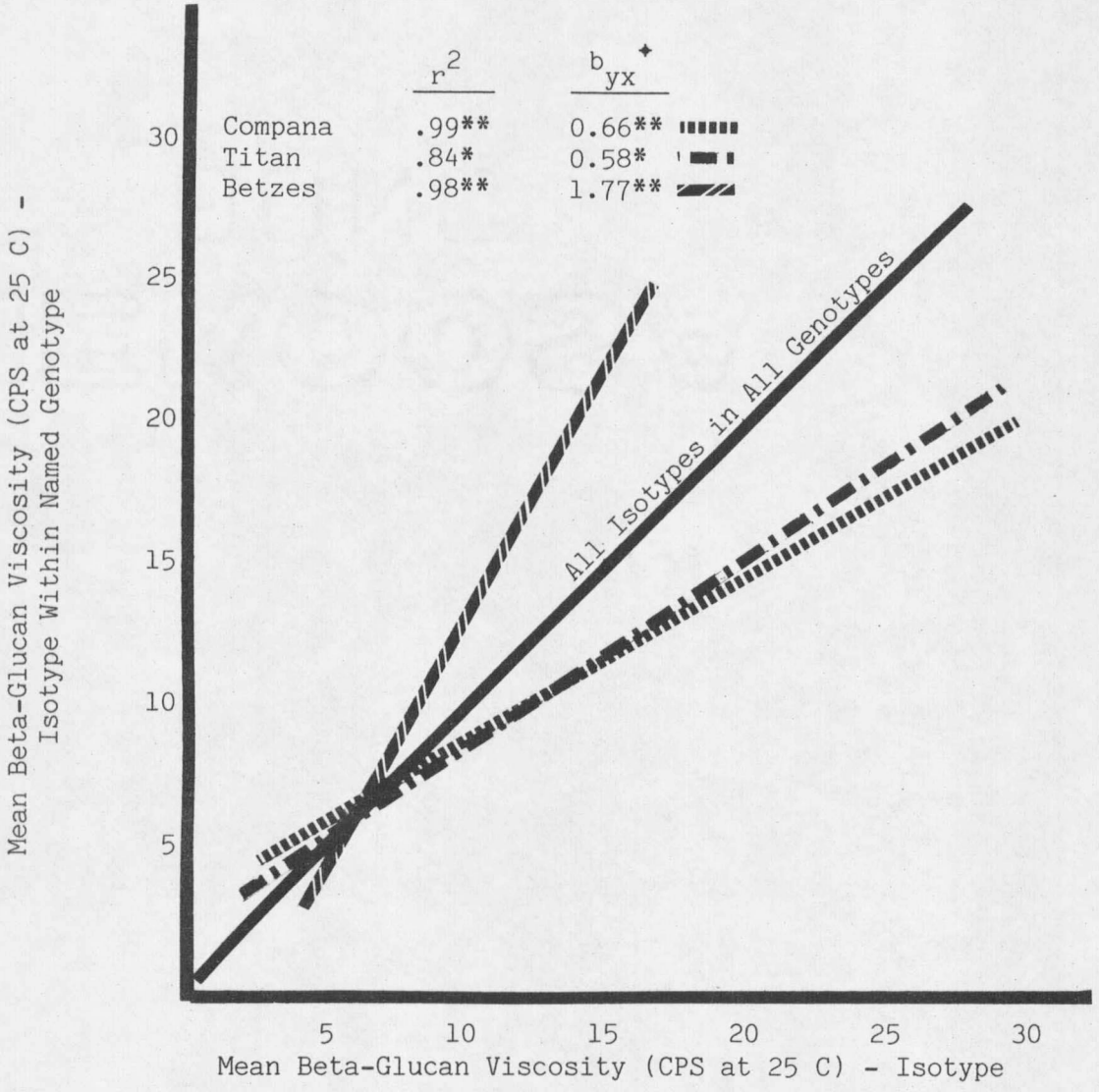

Figure 7-6. Beta-glucan viscosity relationship between the means of six isotypes of Titan, Betzes, and Compana (all environments) and the mean of the six isotypes in all genotypes (all environments).
*, **Significant at the 0.05 and 0.01 levels, respectively.
✦Significant differences from a slope of 1.0 are starred.

magnitude of the response of the three genotypes across isotypes of increasing beta-glucan viscosity.

The r^2 values for the I x E interaction (Figure 7-7) were significant for all five mutant isotypes, however, quite a bit of unassociated variation was observed for the n (1 - .57), wx n (1 - 64), and lk2 n (1 - .75) isotypes. The b_{yx} values for the wx (2.52**) and wx n (4.99*) isotypes were significantly different from the normal isotype.

All the mutant isotypes had b_{yx} values greater than one. This differential response of the isotypes to environments resulting in increased beta-glucan viscosity was the cause of the interaction, with the wx and wx n isotypes having an especially strong influence.

In the isotype within genotype analysis (Table 6-3), significant I x E interactions were observed within all three varieties (Compana, P = .05; Titan and Betzes, P = .01).

The r^2 values for the I x E interaction within Compana (Figure 7-8) were highly significant for all six Compana isotypes. The b_{yx} values for the wx (2.41*), wx n (3.78**), lk2 n (1.94**), and wx lk2 n (7.11**) isotypes were significantly different from the normal isotype. Thus, the differential response of isotypes across environments resulting in increased beta-glucan viscosity was the main source of interaction.

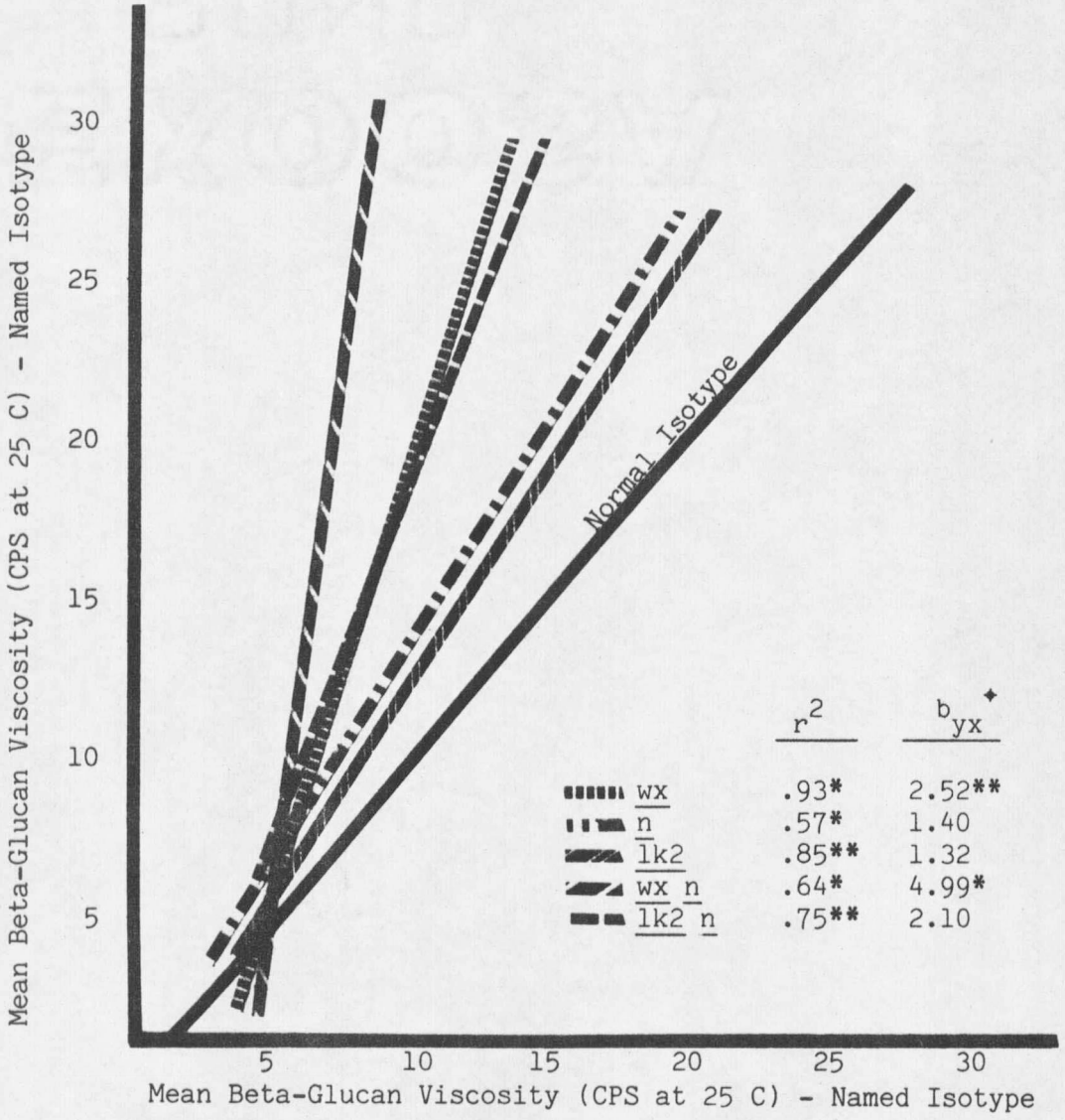

Figure 7-7. Beta-glucan viscosity relationship between the means of five mutant isotypes (all genotypes) and the mean of the normal isotype (all genotypes) grown in eight environments.
*,**Significant at the 0.05 and 0.01 levels, respectively.
✦Significant differences from a slope of 1.0 are starred.

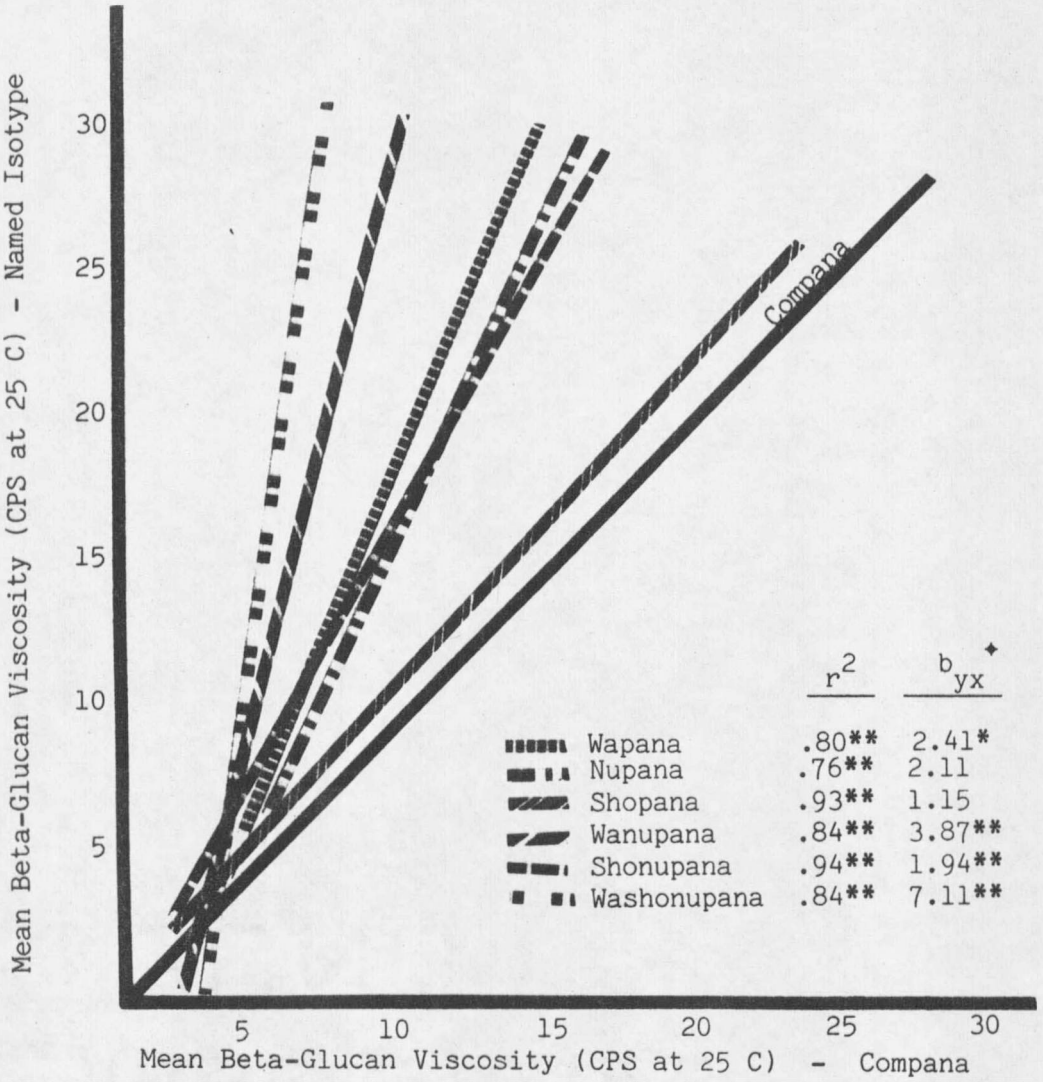

Figure 7-8. Beta-glucan viscosity relationship between the means of six mutant isotypes of Compana and the mean of Compana grown in eight environments.
*,**Significant at the 0.05 and 0.01 levels, respectively.
✦Significant differences from a slope of 1.0 are starred.

The r^2 values for the I x E interaction within Titan (Figure 7-9) were significant for all five Titan isotypes. The b_{yx} value for the wx n isotype (2.80*) was significantly different from the normal isotype. The elevated response of the wx n isotype appeared to be the chief source of interaction. The general tendency for the b_{yx} values of isotypes to exceed one was observed within Titan, however, one isotype, lk2, had a b_{yx} of 0.97.

The r^2 values for the I x E interaction within Betzes (Figure 7-10) were not significant for all isotypes. Unassociated variation was quite high for the n (1 - .39), lk2 n (1 - .55), and wx n (1 - .44) isotypes. While the r^2 values for the wx (.84**) and lk2 (.74**) isotypes were significant, a good bit of unassociated variation was observed for them also. The b_{yx} value for the wx (2.75*) isotype was significantly different from the normal isotype. As observed previously, the isotypes all had b_{yx} values greater than one. The differential response of the isotypes, especially the wx n isotype, was the probable cause of interaction. However, the high levels of unassociated variation could also represent a possible source of interaction.

In order to find a possible source of the three-factor interaction, it was necessary to examine the unassociated variation associated with any isotype across environments within a variety. Such

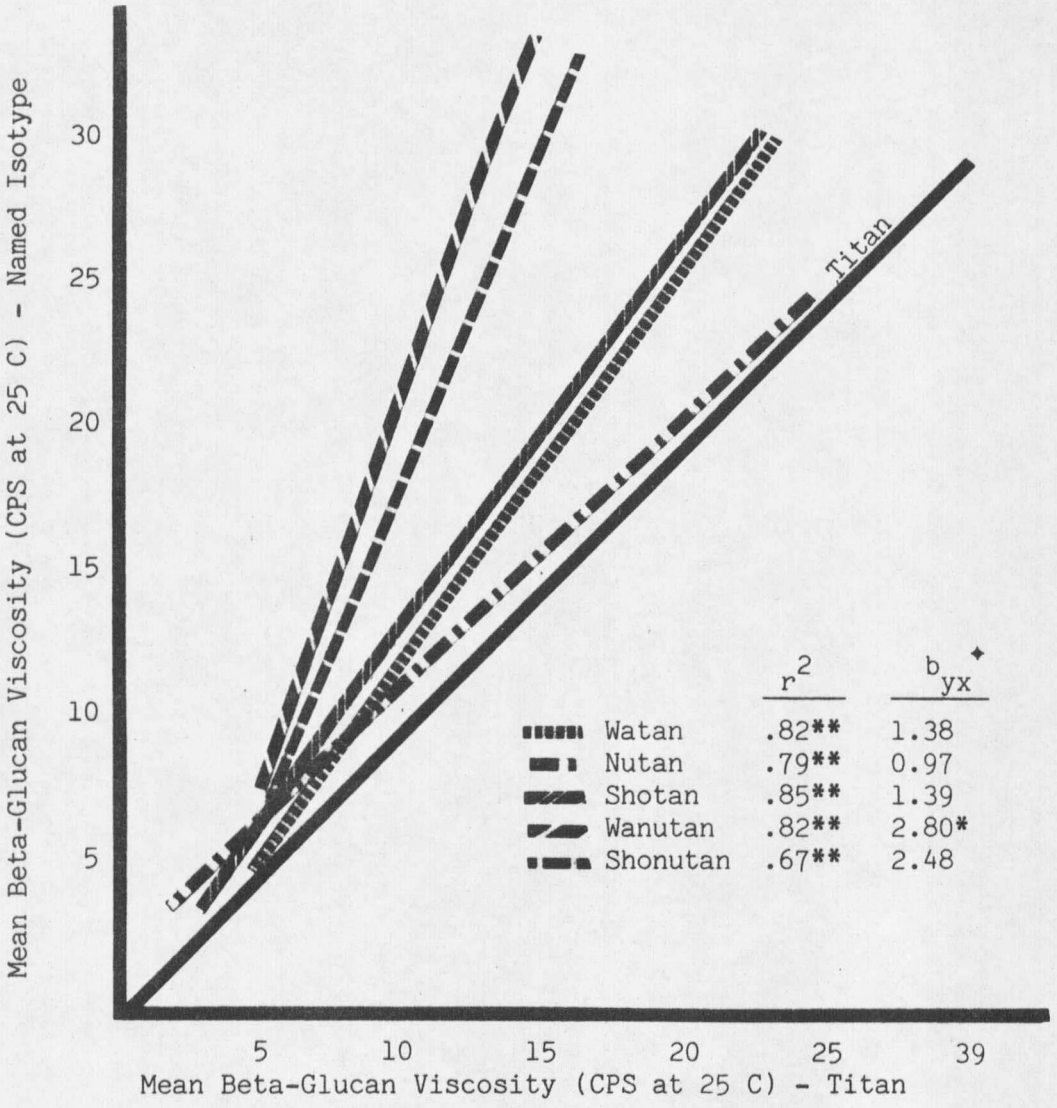

Figure 7-9. Beta-glucan viscosity relationship between the means of five mutant isotypes of Titan and the mean of Titan grown in eight environments.
*, **Significant at the 0.05 and 0.01 levels, respectively.
✦Significant differences from a slope of 1.0 are starred.

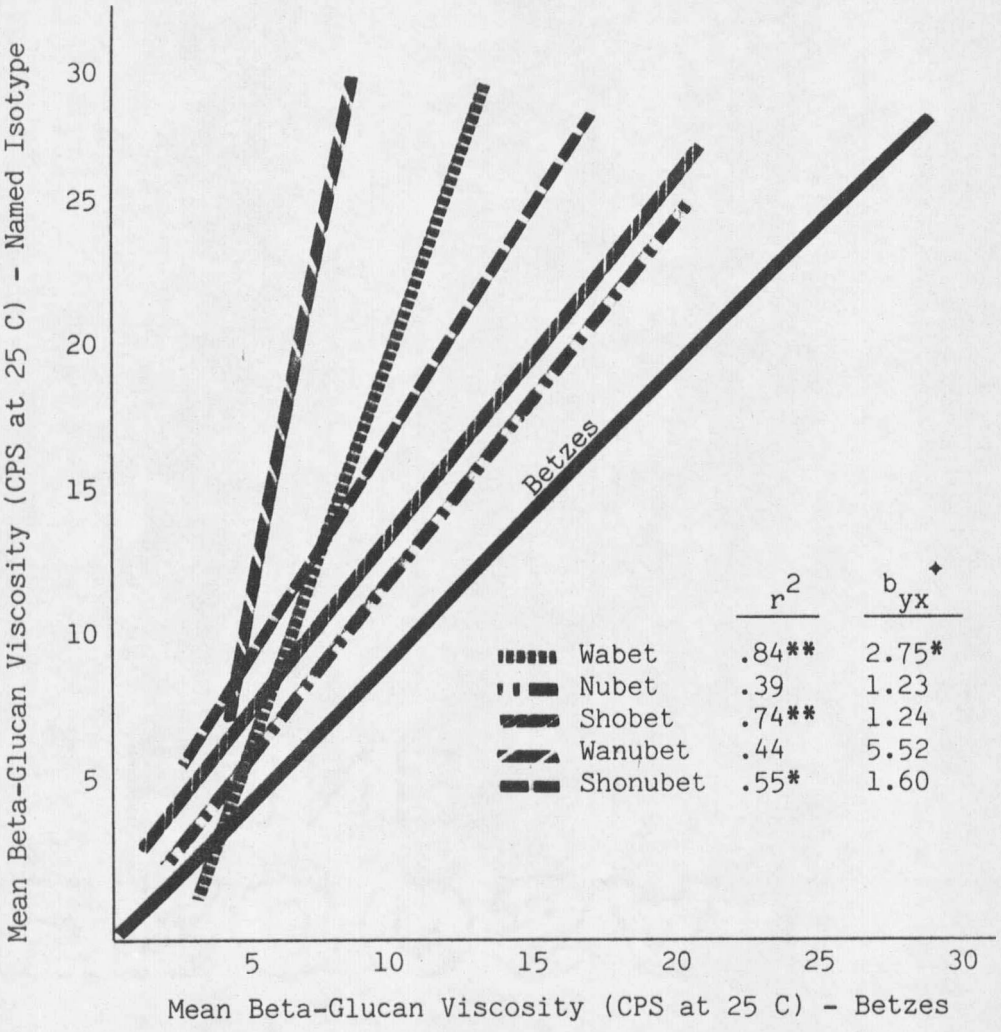

Figure 7-10. Beta-glucan viscosity relationship between the means of five mutant isotypes of Betzes and the mean of Betzes grown in eight environments.
*, **Significant at the 0.05 and 0.01 levels, respectively.
✦Significant differences from a slope of 1.0 are starred.

variation would result from a three-factor combination (G x I x E). The r^2 values for all regressions in Figures 7-8 and 7-9 were significant. However, in the Betzes background (Figure 7-10), the n and wx n isotypes, when examined for beta-glucan viscosity, were not significantly correlated to beta-glucan viscosity of the environment. Thus, all three factors (G x I x E) were necessary to categorize such samples and as such, they represent a three-factor interaction.

Betzes was more severely affected (increased beta-glucan viscosity) when examined across environments and isotypes resulting in increased beta-glucan viscosity. Compana was the genotype least affected by environments of increased beta-glucan viscosity, and Titan was the least affected by isotypes with increased beta-glucan viscosity.

The wx, wx n, and wx lk2 n isotypes were most severely affected by environments resulting in increased beta-glucan viscosity, with the magnitude of the response increasing in the order, wx lk2 n wx n wx. The waxy gene appeared to have a drastic effect upon the interaction between isotype and environment. All the isotypes, however, tended to increase the magnitude of the response of cultivars to environments of increasing beta-glucan viscosity.

The large amounts of unassociated variation observed for the lk2 n and wx n isotypes across increasing beta-glucan environments within

the Betzes genotype strongly indicated a triple interaction in which levels of beta-glucan viscosity were determined by specific genotype x isotype x environment combinations.

Percent Extract

The combined analysis of variance (Table 6-2) showed that the I x E interaction was significant for predicted percent extract. The r^2 values for this interaction (Figure 7-11) were significant for all five mutant isotypes. No b_{yx} values significantly different from one or from each other were observed. Thus, the significant interaction is possibly due to the cumulative effect of all the b_{yx} deviations from one.

In the isotype within genotype analysis (Table 6-3), a significant I x E interaction was observed within Titan ($P = .01$). The r^2 values for this interaction (Figure 7-12) were significant for each isotype. No b_{yx} value was significantly different from one or from each other. The interaction is likely due to the cumulative effect of all b_{yx} deviations from one.

In general, the predicted percent extract response variable was not highly interactive with the three factors examined in this study.

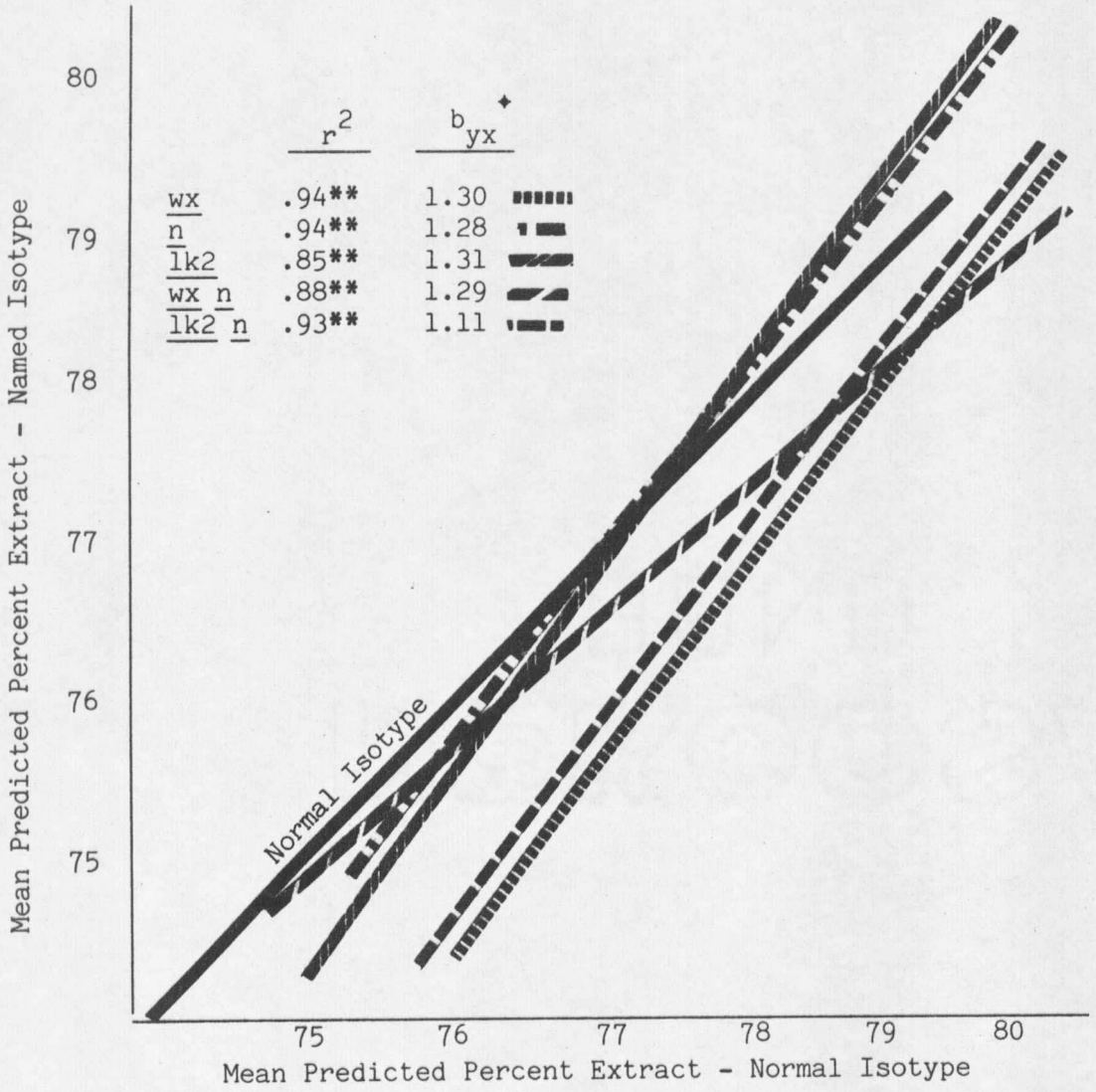

Figure 7-11. Predicted percent extract relationship between the means of five mutant isotypes (all genotypes) and the mean of the normal isotype (all genotypes) grown in eight environments.
**Significant at the 0.01 level.
✦Significant differences from a slope of 1.0 are starred.

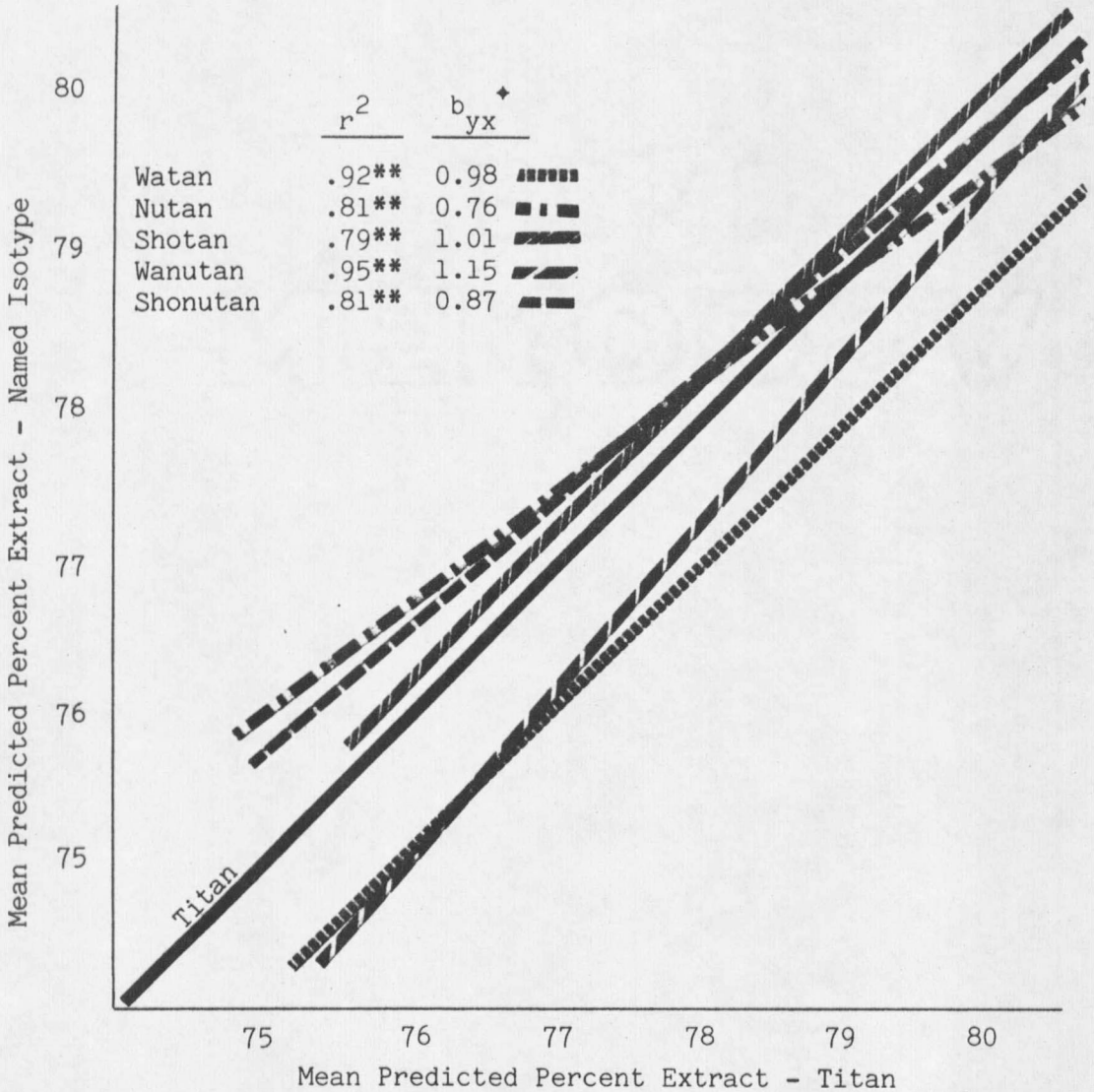

Figure 7-12. Predicted percent extract relationship between the means of five isotypes of Titan and the mean of Titan grown in eight environments.
**Significant at the 0.01 level.
✦Significant differences from a slope of 1.0 are starred.

Alpha-Amylase Activity

The combined analysis of variance (Table 6-2) showed that the G x E interaction was significant for alpha-amylase activity.

The r^2 values for this interaction (Figure 7-13) were significant for Titan only. The b_{yx} values for Titan (2.52**) and Compana (0.08**) were significantly different from one. The vastly different behavior of the three genotypes across environments of increasing alpha-amylase activity was the principle cause of the interaction. However, a large amount of unaccounted variability was also present in Compana (1 - .04) and Betzes (1 - .36). This, also, can have an appreciable effect on the interaction. These results indicate that no consistent environmental gradient for alpha-amylase in nongerminated seed exists which can be applied to the three genotypes examined.

Summary

Regression analysis provided information on the interactions among isotype, genotype, and environment, for kernel weight. With respect to genotypes, it was found that Compana, Betzes, and Titan responded to favorable environmental conditions for kernel development, in that order, greatest to least. When examined across increasing kernel weight isotypes, Compana, Titan, and Betzes responded to favorable isotypes, in that order, greatest to least.

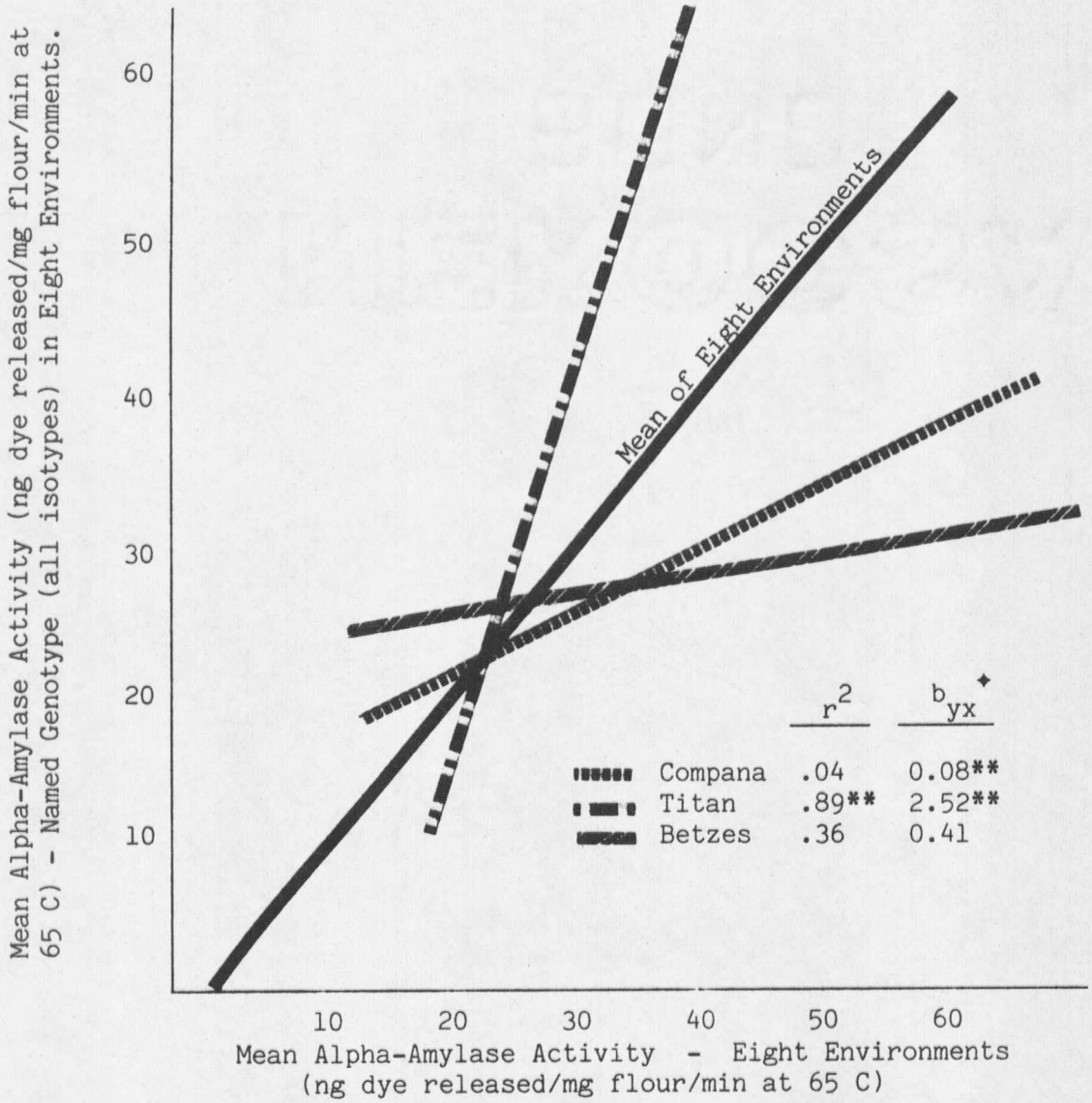

Figure 7-13. Alpha-amylase activity relationship between the means of Titan, Betzes, and Compana (all isotypes) and the mean of all entries grown in eight environments.
**Significant at the 0.01 level.
✦Significant differences from a slope of 1.0 are starred.

All of the mutant isotypes, except lk2, were found to not only produce seeds of lower kernel weight when compared to normal, but were less able to utilize environments favoring grain filling than the normal isotypes. These results indicate that when certain traits, which in general reduce kernel weight, are being utilized in a breeding program, their effect can be greatly dampened or accentuated by the background genotype into which they are incorporated.

Regression analysis provided information on the interactions among isotype, genotype, and environment, for beta-glucan viscosity. With respect to genotypes, it was found that when environments were favorable for beta-glucan viscosity development, Betzes, Titan, and Compana responded in that order, greatest to least. When Betzes, Compana, and Titan were examined across isotypes with increasing beta-glucan viscosity, they responded in that order, greatest to least.

All of the mutant isotypes had a beta-glucan viscosity response greater than the normal type, but only the waxy isotypes were significant. Within the waxy isotypes, the beta-glucan viscosity response across environments increased in the order: wx lk2 n > wx n > wx. As with kernel weight, the beta-glucan viscosity regression analysis results indicated that by manipulating the isotype and background

genotype, the plant breeder can produce cultivars with an extensive range of beta-glucan viscosities.

While some interactions among isotype, genotype, and environment were observed for the response variables percent protein, predicted percent extract, and alpha-amylase activity, they were not of sufficient magnitude for the regression analysis to provide a clear indication of the source of the interactions. No pronounced trends were observed that could be used in a breeding program.

CHAPTER 8

A RAPID SCREENING METHOD FOR BARLEY CULTIVARS SUITABLE FOR SYRUP PROCESSING

Introduction

The Brabender Amylograph has been used to characterize barley cultivars for syrup production. The optimum barley grain for processing has a pasting peak temperature between 65-75 C and a low pasting peak viscosity. Studies have shown that these two factors are affected by alpha-amylase and beta-glucanase (implying the presence of beta-glucans). Previous studies in this dissertation indicate that both alpha-amylase and beta-glucans are affected by genotype, isotype, and environment.

The purpose of the research presented in this section was to 1) characterize all of the available waxy isotypes of Betzes, Titan, and Compana, with respect to pasting peak and pasting peak temperature, and 2) develop a method of predicting these two characteristics rapidly and using small samples.

Materials and Methods

Single samples of four waxy isotypes of the cultivars Betzes, Titan, and Compana were used in this study. The isotypes included

were the wx, wx n, wx lk2, and wx lk2 n. A discussion of the derivation and naming of these isotypes can be found in Chapter 2. Seven of the samples were grown as increases in Bozeman, Montana, 1979: Wapana, Wanupana, Washonupana, Watan, Wanutan, Wabet, and Wanubet. The remaining five samples were grown as increases in Mesa, Arizona, 1970-1980 winter nursery: 'Washopana', 'Washotan', 'Washonutan', 'Washobet', and 'Washonubet'.

Brabender curves were determined using a technique described by Goering et al. (1981, see acknowledgements). Beta-glucan viscosities were determined using the method described in Chapter 6. Alpha-amylase determinations were performed using the BPA assay described in Chapter 3 and converted to CBA units as described in Study IV of Chapter 3.

The isotype x cultivar interaction was used in an F-test to determine the significance of the two main effects, isotype and cultivar, upon pasting peak viscosity, beta-glucan viscosity, and alpha-amylase activity. Regression analyses were performed to relate beta-glucan viscosity and alpha-amylase activity to pasting peak viscosity. All units were transformed to $\log_e$.

Results and Discussion

Pasting Peak Temperature and Viscosity

All isotypes within the three cultivars pasted at about the same temperature, from 65-75 C (Figures 8-1, 8-2, and 8-3). Thus, it appears that all waxy isotypes and genotypes behave similarly with respect to pasting temperature. The pasting peak viscosity was different. Table 8-1 shows this character was significantly influenced by background genotype and isotype. Betzes isotypes (Figure 8-3) had extremely high pasting peaks (i.e., high viscosity), while Compana (Figure 8-1) and Titan (Figure 8-2) were considerably lower with Titan being somewhat lower than Compana.

The isotype effect was observed across all three cultivars. The wx lk2 n isotype had the highest pasting peak viscosity, and the wx isotype had the lowest. The wx lk2 and wx n were intermediate to the other two, in that order. The means of the cultivars and isotypes are listed in Table 8-2.

Association Between Pasting Peak, Beta-Glucan Viscosity, and Alpha-Amylase Activity

Previous research with barley flour samples examined in a Brabender Amylograph (Yamada and Yoshida, 1973) has indicated that the presence of small amounts of alpha-amylase in the barley flour sample

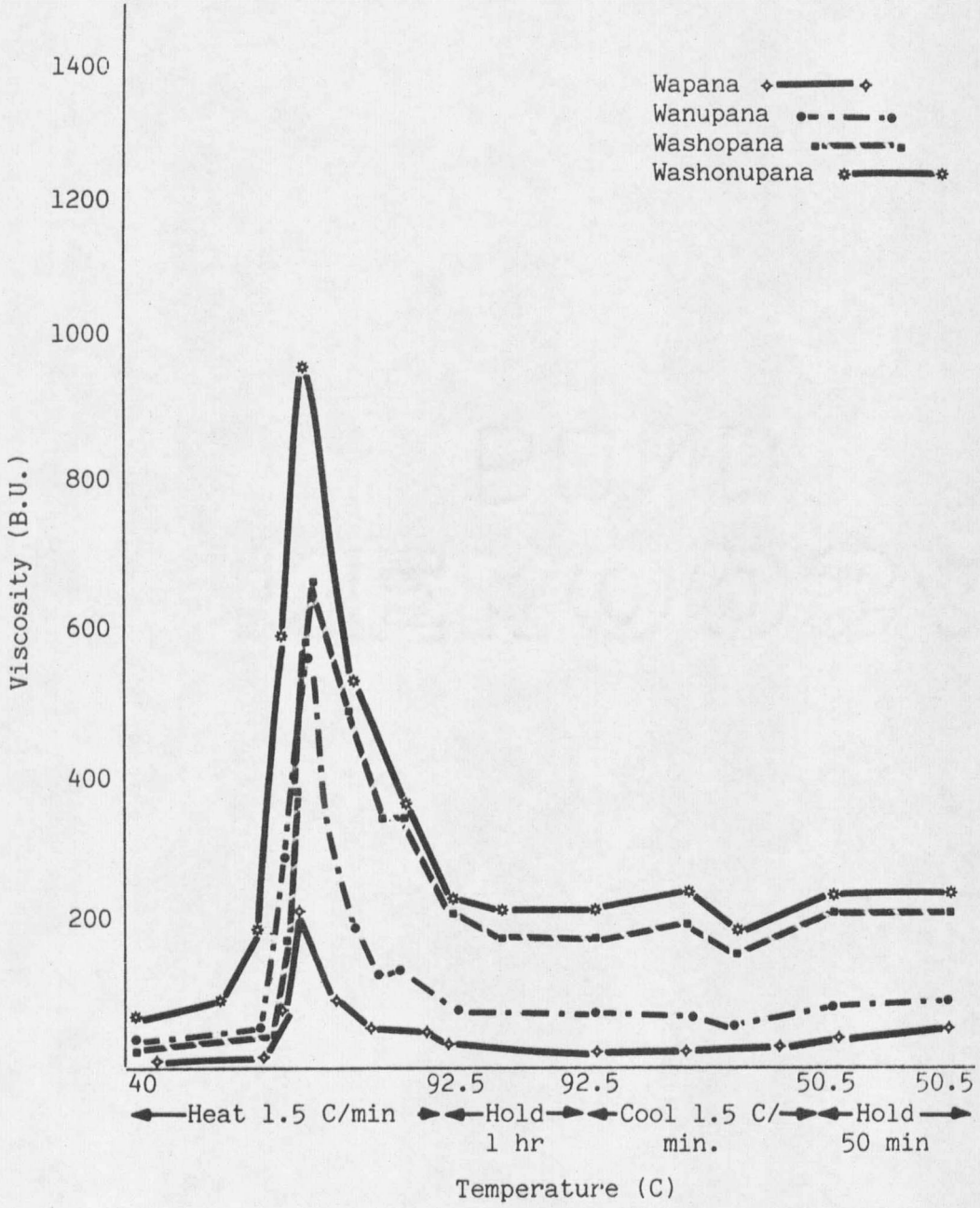

Figure 8-1. Brabender amylograms of flour samples of four waxy isotypes of Compana.

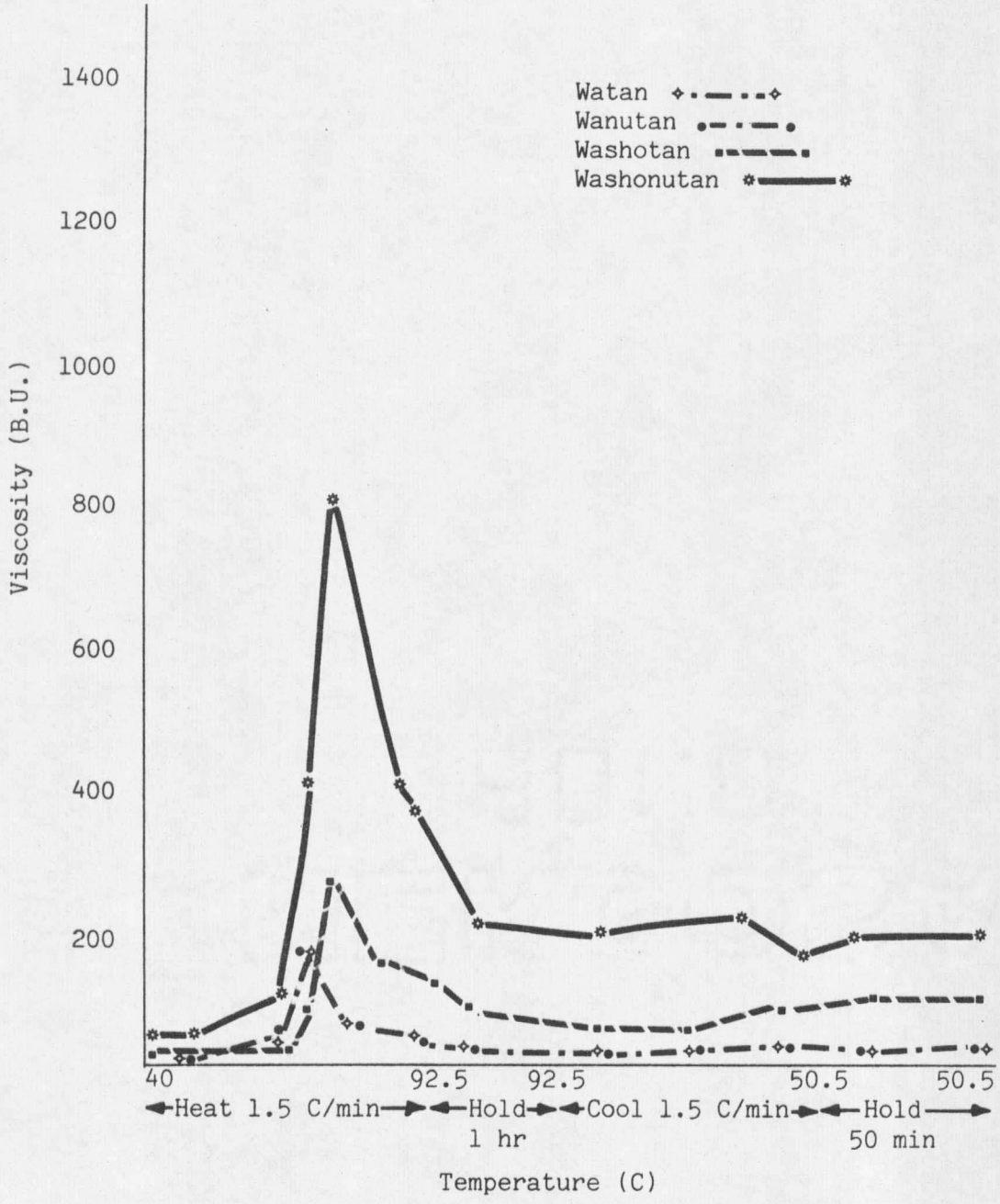

Figure 8-2. Brabender Amylograms of flour samples of four waxy isotypes of Titan.

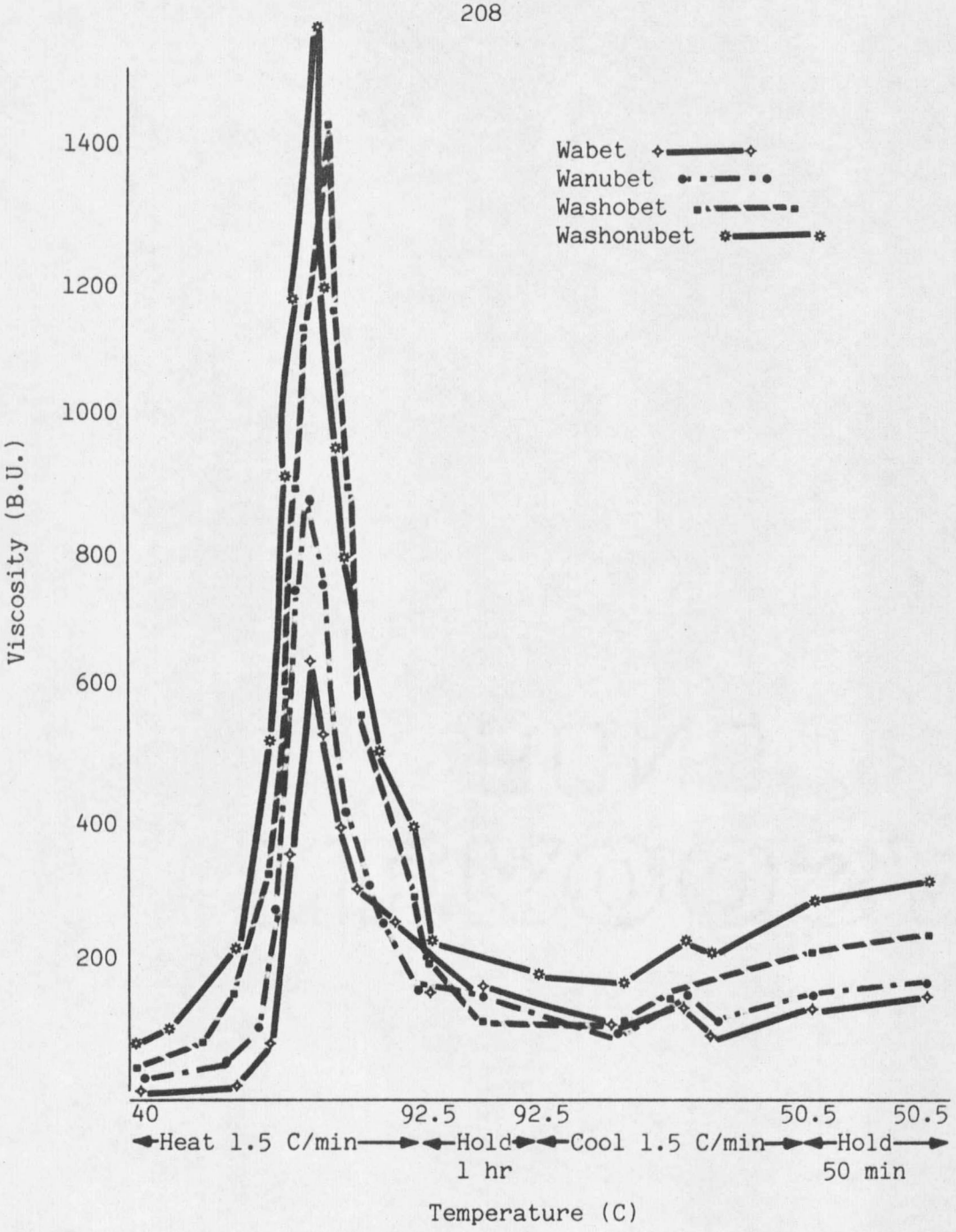

Figure 8-3. Brabender Amylograms of flour samples of four waxy isotypes of Betzes.

Table 8.1 Analysis of variance for pasting peak viscosity, beta-glucan viscosity, and alpha-amylase activity of unreplicated samples of four waxy isotypes within three cultivars.

Source of Variance	df	Mean Square		
		Pasting Peak Viscosity (B.U.)	Beta-Glucan Viscosity (CPS at 25 C)	Alpha-Amylase Activity (ng dye released/mg flour/min at 65 C)
Isotype (I)	3	354541.67**	1032.98	85.60
Cultivar (C)	2	651025.00**	1436.14*	185.55*
I x C	6	25425.00	251.33	29.68

*, **Significant at the 0.05 and 0.01 levels, respectively.

Table 8-2. Mean pasting peak viscosity, beta-glucan viscosity, and alpha-amylase activity of three cultivars (across four isotypes) and four isotypes (across three cultivars).

Cultivar or Isotype	n	Mean		
		Pasting Peak Viscosity (B.U.)	Beta-Glucan Viscosity (CPS at 25 C)	Alpha-Amylase Activity (ng dye released/mg flour/min at 65 C)
Cultivar				
Betzes	4	1132.5	54.2	21.7
Compana	4	605.0	29.8	22.2
Titan	4	340.0	16.9	33.7
$L.S.D._{0.05}$		275.9	27.4	9.4
Isotype				
wx	3	340.0	17.8	31.3
wx n	3	526.7	38.0	29.4
wx lk2	3	766.7	20.7	22.4
wx lk2 n	3	1136.7	58.0	20.3
$L.S.D._{0.05}$		318.6	31.7	10.9

greatly reduced pasting peak viscosity. In addition, the unmodified beta-glucan found in barley flour tended to increase pasting peak viscosity.

The means of cultivars and isotypes in Table 8-2 show that the same general trend is being observed in this material. The samples with the highest alpha-amylase activity have the lowest Brabender peak viscosity. Conversely, the samples of highest beta-glucan viscosity have the highest Brabender peak viscosity.

In order to test these general relationships, three regressions were calculated: 1) $\log_e$ Brabender pasting peak viscosity vs. $\log_e$ alpha-amylase activity, 2) $\log_e$ Brabender pasting peak viscosity vs. $\log_e$ beta-glucan viscosity, and 3) $\log_e$ Brabender pasting peak viscosity vs. $\log_e$ of alpha-amylase activity and beta-glucan viscosity.

The relationship between alpha-amylase activity and pasting peak viscosity was highly significant for the 12 samples tested ($r = .90^{**}$) $b_{yx} = -2.48$) as seen in Figure 8-4. The relationship between beta-glucan viscosity and Brabender pasting peak viscosity was also highly significant for the 12 samples tested ($r = .85^{**}$, $b_{yx} = 0.88$) as seen in Figure 8-5. The multiple regression of $\log_e$ Brabender pasting peak viscosity vs. $\log_e$ alpha-amylase activity (x_1) and $\log_e$ beta-glucan viscosity (x_2) is highly significant ($r = .96^{**}$; $Y = 10.06 - 0.86\ x_1 + 0.79\ x_2$) as seen in Figure 8-6. Thus, these two factors

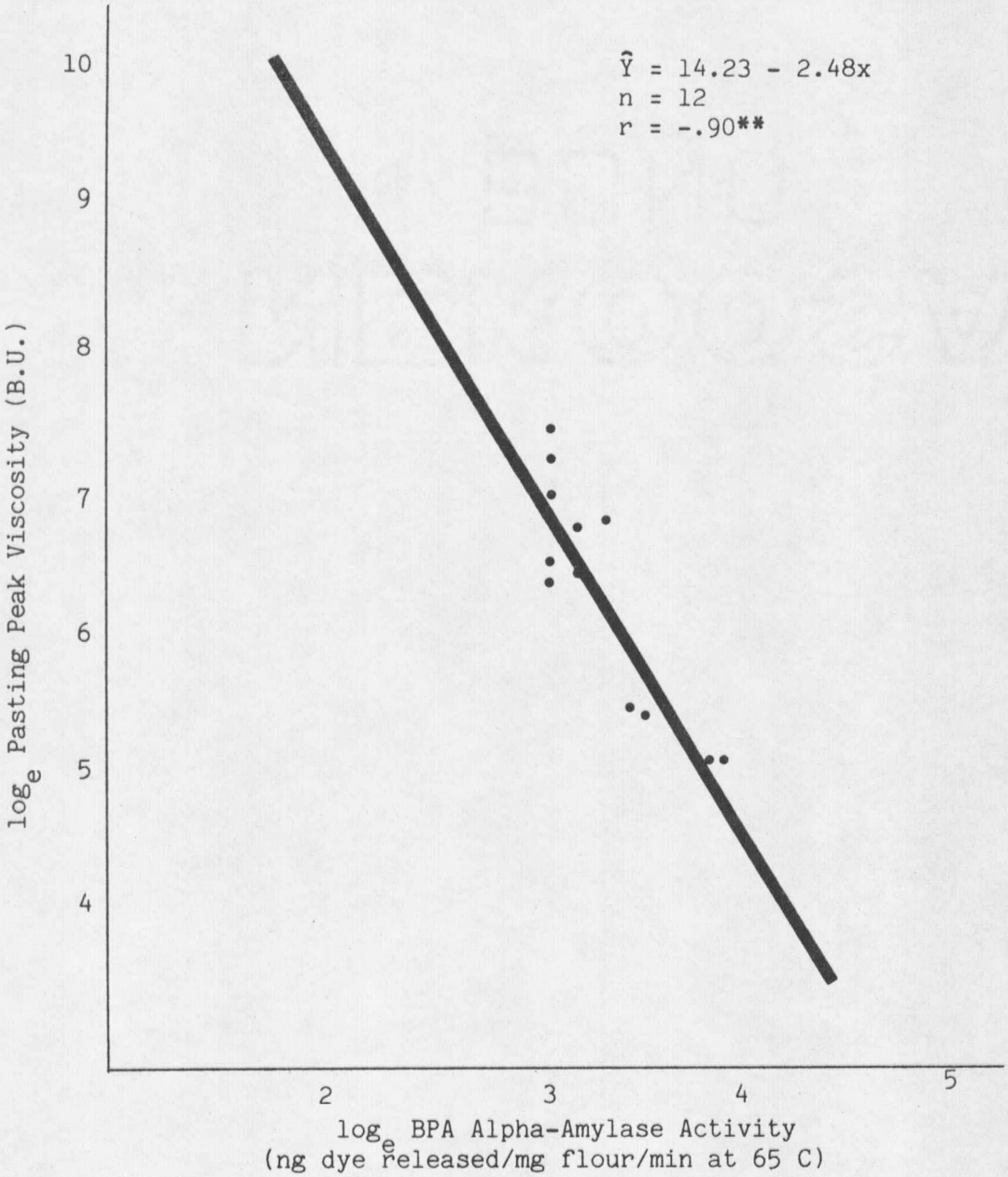

Figure 8-4. Regression of log$_e$ pasting peak viscosity to log$_e$ BPA alpha-amylase activity for four waxy isotypes of Betzes, Compana, and Titan.
**Significant at the 0.01 level.

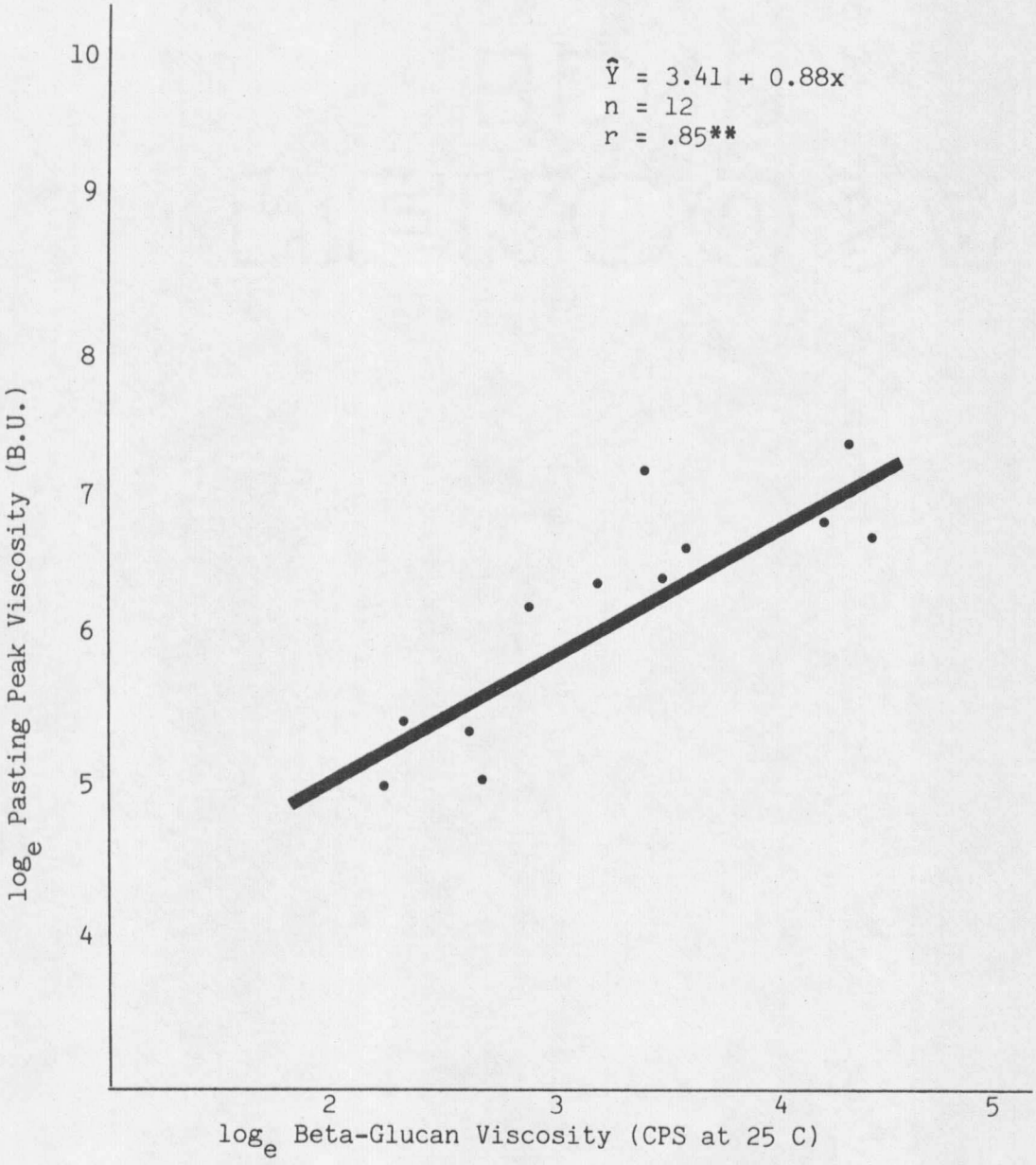

Figure 8-5. Regression of log$_e$ pasting peak viscosity to log$_e$ beta-glucan viscosity for four waxy isotypes of Betzes, Compana, and Titan.
**Significant at the 0.01 level.

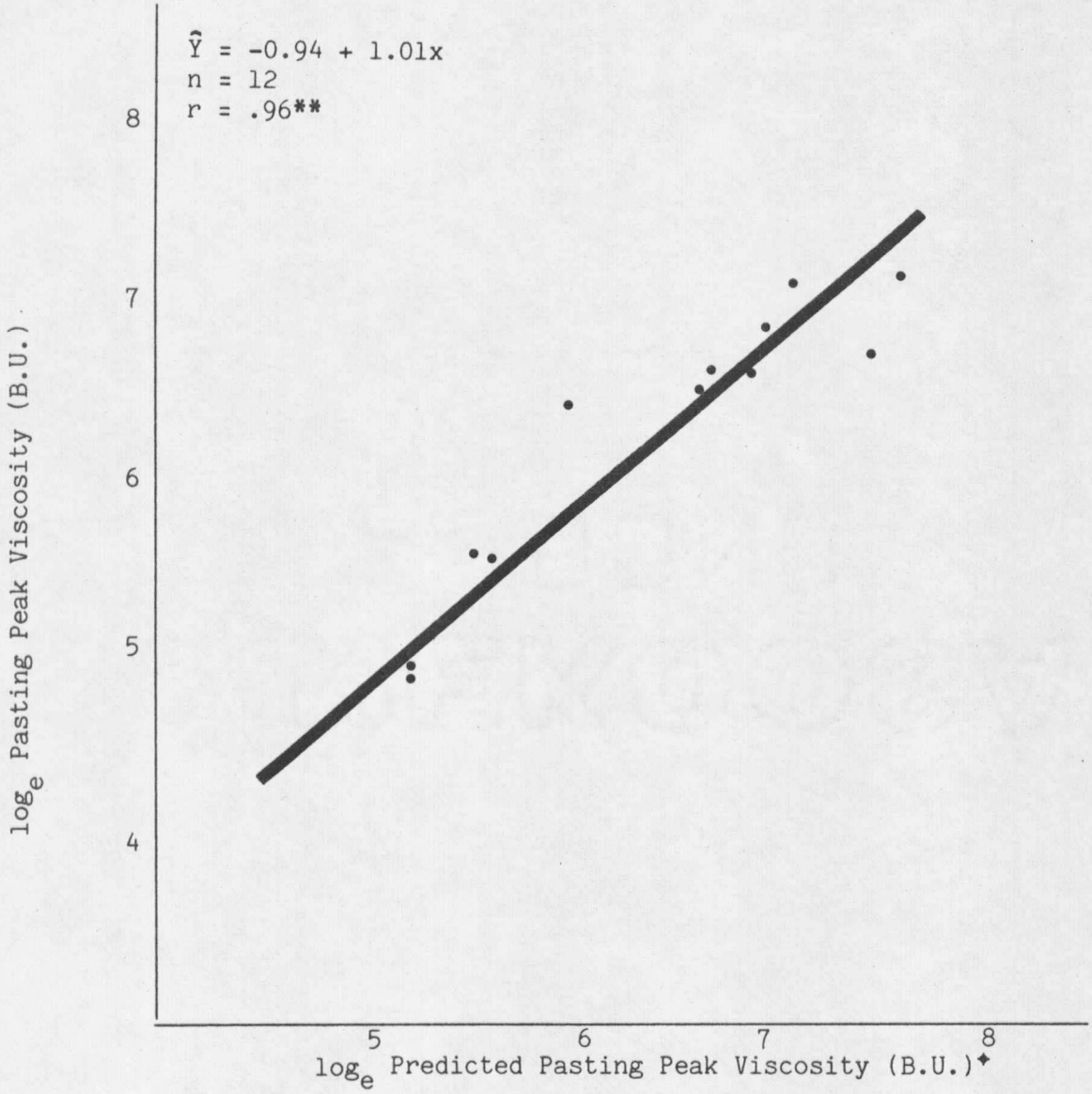

Figure 8-6. Relationship between actual $\log_e$ pasting peak viscosity and a predicted $\log_e$ pasting peak viscosity.
✦Predicted using the equation $\hat{Y} = 10.06 - 0.86x_1 + 0.79x_2$ based on alpha-amylase activity (x_1) and beta-glucan viscosity (x_2).
**Significant at the 0.01 level.

explain almost 93% of the variability associated with the Brabender pasting peak viscosity of the 12 waxy barley flour samples.

The data reported in Chapter 6 demonstrated that environment, cultivar, and isotype all have significant effects on beta-glucan viscosity and alpha-amylase activity in barley. Thus the pasting peak viscosity values observed in a Brabender Amylograph can have at least three sources of variation. The environment can have an especially drastic effect on alpha-amylase activity since barley exposed to moisture at harvest will begin to produce large amounts of this enzyme (sprout damage). Thus, a sprouted barley sample of a particular cultivar will produce a very different peak viscosity than an unsprouted sample of the same variety. Yamada and Yoshida (1973) demonstrated that with addition of alpha-amylase not only is pasting peak viscosity reduced, but the actual pasting temperature is reduced.

Data from Chapter 6 support trends observed in this section. First, Betzes beta-glucan viscosity was much higher than Titan or Compana. The pasting peak viscosity for Betzes was much higher than that of Titan or Compana. Second, the beta-glucan viscosity increased across isotypes in the following order: wx lk2 n > wx n > wx. This same relationship was observed for pasting peak viscosity.

Third, it was observed that alpha-amylase activity of Titan was higher than that of Compana or Betzes. Peak pasting viscosity was observed to be the lowest in Titan isotypes.

Brabender Amylographs are too time consuming for use as a screening method as only two flour samples a day can be tested. A much quicker method would be to measure alpha-amylase activity and beta-glucan viscosity for barley flour samples and use a prediction equation to estimate peak pasting viscosity. In this study 93% of the variation among barley samples was accounted for by this method (50 to 100 samples could be screened daily).

Summary

All waxy cultivars and isotypes within cultivars pasted in the same temperature range (65-75 C) in a Brabender Amylograph. Pasting peak viscosity, however, was observed to be different for cultivars and isotypes within cultivars. Betzes isotypes had extremely high pasting peak viscosities while Compana and Titan isotypes were considerably lower with Titan isotypes somewhat lower than Compana. A similar isotype effect was observed across all three cultivars. The wx lk2 n isotypes had the highest pasting peak viscosities followed by the wx lk2, wx n, and wx isotypes.

The $\log_e$ of Brabender peak pasting viscosity for the 12 samples used in this study was found to be related to $\log_e$ alpha-amylase activity of samples ($r = .90^{**}$; $b_{yx} = -2.48$) and to $\log_e$ beta-glucan viscosity of samples ($r = .85^{**}$; $b_{yx} = 0.88$). When $\log_e$ peak pasting viscosity is related to both $\log_e$ alpha-amylase activity and $\log_e$ beta-glucan viscosity by multiple regression, an excellent multiple correlation is obtained ($r = .96^{**}$).

These results indicate that for rapid screening purposes the alpha-amylase activity and the beta-glucan viscosity of a small barley flour sample can be used in a prediction equation to estimate Brabender pasting peak viscosity with a great deal of accuracy and a great saving to time and amount of sample.

CHAPTER 9

SUMMARY AND CONCLUSIONS FROM ALL STUDIES

A rapid but accurate assay for alpha-amylase was developed so that alpha-amylase activity could be determined in a timely and efficient manner for large numbers of barley flour samples. The Blue Plate Agar (BPA) assay is a gel-diffusion assay in which a chromogenic substrate was suspended in an agar medium. Alpha-amylase activity was measured as the diameter of a digestion circle that develops around sample wells containing barley flour/water paste. The area of each digestion circle was highly correlated to the $\log_{10}$ of alpha-amylase activity as measured by the IDC assay ($r = .89^{**}$), CBA assay ($r = .99^{**}$), and Degrees Lintner Reducing Power ($r = .99^{**}$).

The assay was accurate across the 25-65 C temperature range. Optimum sensitivity was at the 50-55 C which is also the temperature optimum for alpha-amylase. Incubation times as short as 1 hour were used effectively at 65 C but the accuracy of the assay increased with incubation time. Four hour incubation at 55 C or 65 C proved to be an excellent regimen.

The BPA assay accurately measured the entire range of enzyme activity from the minute amounts found in ripe, nongerminated seed, to

the amounts found in malt, and malt amylase extracts. This assays capability to detect 0-6% sprout damage, and to distinguish between malt and feed cultivars before and after malting, indicates it would be useful in breeding programs which require a quick, simple, inexpensive, but accurate means to measure alpha-amylase.

Very small amounts of alpha-amylase were found in all samples of 50 commercial cultivars grown in several environments. No unique levels or isozymes of alpha-amylase were found in Washonupana or any other samples of waxy, short-awn, or hulless barley except in those samples that exhibited post-harvest sprout damage.

The waxy starch found in Washonupana as well as the waxy isotypes of Compana, Titan, Betzes, and Oderbrucker proved to be 2-3 times more susceptible to digestion by alpha-amylase present in ripe seed than normal starch. Thus, the self-liquefying property observed for Washonupana starch appears to result from an interaction between the digestible waxy starch and the small amounts of alpha-amylase found to varying degrees in all barley at harvest.

Upon malting, waxy endosperm isotypes within Compana, Titan, and Betzes produced significantly more reducing substances (maltose) than nonwaxy types while producing the same level of alpha-amylase and for at least one variety (Compana) significantly lower levels of beta-

amylase. This phenomenon can only be explained by the extremely digestible nature of the waxy starch. The only difference between genotypes was in the degree to which the waxy isotypes exceeded the normal for reducing substances after malting (Betzes 13%, Compana 22%, Titan 28%).

With the addition of the waxy endosperm, short-awn, and hulless seed, kernel weights were reduced in all three genotypes. The order of isotypes from high to low kernel weight was: normal > waxy endosperm > short-awn > hulless > waxy endosperm, hulless > short-awn, hulless > waxy, short-awn, hulless (evaluated in Compana background only).

The beta-glucan viscosity was inversely related to kernel weight for nonwaxy isotypes ($r = .97^*$). However, waxy isotypes had beta-glucan viscosity significantly greater than that predicted by kernel weight alone. This indicated that viscosity was increased in waxy barley isotypes beyond a simple beta-glucan concentration effect due to kernel weight reduction.

Beta-glucans could possibly serve as an alternate storage polysaccharide in waxy barley. This supposition is supported by the fact that when examined for predicted percent extract (an indirect measurement of starch content) waxy isotypes were significantly lower than normal

types while percent protein was significantly higher. If starch synthesis is reduced in waxy isotypes, beta-glucan synthesis would serve as a convenient alternate pathway to store carbohydrates.

The kernel weight and beta-glucan viscosity response of waxy-endosperm, short-awn, and hulless seed isotypes were significantly influenced by environment and genotype. The kernel weight reductions associated with each isotype were most severe within the two-row, large seeded Compana background and least severe in the six-row Titan background with Betzes being intermediate. As the individual isotypes were examined across environments it was found that, with the exception of the short-awn isotype, they were less able to utilize environments favoring grain filling than normal types.

The response of each isotype to beta-glucan viscosity was most severe (high beta-glucan viscosity) in the Betzes background while the response in Compana and Titan was much lower, in that order. The response of the isotypes to environments of increasing beta-glucan viscosity was marked by a higher rate of increase for all isotypes when compared to the normal with the response rate of waxy isotypes significantly higher than the normal types.

When waxy, waxy hulless, waxy short-awn, and waxy short-awn hulless isotypes within Compana, Titan, and Betzes, were evaluated

in a Brabender Amylograph, it was found that all pasted in the same temperature range (65-75°C). However, the pasting peak viscosity was significantly affected by genotype and isotype. It was found that the pasting peak viscosity was related negatively to alpha-amylase content and positively to beta-glucan viscosity of a barley flour sample (r^2 = .93**). Thus, due to high beta-glucan viscosities, Betzes isotypes had higher pasting peak viscosities than Titan or Compana. Titan isotypes had the highest alpha-amylase levels and also lowest viscosities. The following relationship was observed for pasting peak viscosity of isotypes: waxy, short awn, hulless > waxy, short awn > waxy, hulless > waxy. The same relationship was observed for beta-glucan viscosity.

The purpose of this research was to evaluate the waxy endosperm, short-awn, and hulless seed genes, singly and in combination, within several genotypes, to ascertain their usefulness in industrial uses and processes. The results of the studies indicated that the waxy endosperm gene alone, imparted the most useful characteristics (highly digestible starch with a low pasting temperature range, 65-75 C) to all genotypes. The inclusion of this gene also resulted in reduced kernel weight and elevated beta-glucan viscosity. These characteristics are not presently regarded as desirable features for

industrial processing. The presence of the short-awn and hulless-seed genes intensified these characteristics. However, the results also indicated that these effects could be accentuated or dampened by the background genotype into which the genes are incorporated.

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APPENDICES

APPENDIX I

Expected Mean Squares for Waxy Isogenic Analysis Grown in 1979

Source of Variation	df	Expected Mean Squares
Environment (E)	7	$\sigma^2_e + 18\sigma^2_{B/E} + 36\sigma^2_E$
Blocks/Environment (B/E)	16	$\sigma^2_e + 18\sigma^2_{B/E}$
Genotypes (G)	2	$\sigma^2_e + 6\sigma^2_{(B/E)G} + 18\sigma^2_{GE} + 144\sigma^2_G$
G x E	14	$\sigma^2_e + 6\sigma^2_{(B/E)G} + 18\sigma^2_{GE}$
(B/E) x G	32	$\sigma^2_e + 6\sigma^2_{(B/E)G}$
Isotype (I)	5	$\sigma^2_e + 9\sigma^2_{IE} + 72\sigma^2_I$
I x E	35	$\sigma^2_e + 9\sigma^2_{IE}$
I x G	10	$\sigma^2_e + 3\sigma^2_{IGE} + 24\sigma^2_{IG}$
I x G x E	70	$\sigma^2_e + 3\sigma^2_{IGE}$
(B/E) x I x (B/E) x G x I	240	σ^2_e

APPENDIX II

Field Design for Waxy Isogenic Analysis Grown in 1979

Replication 1	Replication 2	Replication 3
Black Betzes	Black Betzes	Black Betzes
Shopana	Shonubet	Watan
Wapana	Wabet	Wanutan
Washonupana	Betzes	Shotan
Shonupana	Shobet	Shonutan
Compana	Wanubet	Titan
Wanupana	Nubet	Nutan
Nupana	Black Betzes	Black Betzes
Black Betzes	Wanutan	Shonubet
Wanutan	Shotan	Shobet
Shotan	Shonutan	Nubet
Shonutan	Titan	Wanubet
Watan	Watan	Betzes
Nutan	Nutan	Wabet
Titan	Black Betzes	Black Betzes
Black Betzes	Compana	Piroline
Nubet	Shopana	Gus
Shonubet	Nupana	Shabet
Wabet	Shonupana	Summit
Shobet	Wapana	Klages
Wanubet	Washonupana	Black Betzes
Betzes	Wanupana	Nupana
Black Betzes	Black Betzes	Wapana
Klages	Gus	Washonupana
Gus	Piroline	Compana
Summit	Klages	Wanupana
Piroline	Summit	Shonupana
Shabet	Shabet	Shopana
Black Betzes	Black Betzes	Black Betzes

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