# EFFECT OF GERMINATION AND DRYING ON ENZYME ACTIVITY IN SORGHUM AND COWPEAS

by

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Thesis submitted to the Graduate Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

MASTER'S OF SCIENCE

in

Food Science and Technology

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November, 1994
Blacksburg, Virginia

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# EFFECT OF GERMINATION AND DRYING TIME ON ENZYME ACTIVITY IN SORGHUM AND COWPEAS

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## (ABSTRACT)

Malts prepared from sorghum and cowpeas that was germinated at 25°C for 0, 1, 2 or 3 days and dried at 60°C for either 2.5, 5, 7.5 and 10 hours, were evaluated for $\alpha$-amylase, ß-amylase and endo-(1,3)(1,4)-ß-D-glucanase activity. Germination had a highly significant effect on enzyme activity ($P < 0.01$). However, cowpea endo-(1,3)(1,4)-ß-D-glucanase was not significantly affected by the combined action of germination and drying. ß-Amylase was not detected in any of the samples of this experiment. Further analysis using Duncan's Multiple Range Test, indicated that there was no significant difference in $\alpha$-amylase activity in samples dried between 5 to 10 hours. Compared to $\alpha$-amylase, sorghum and cowpea samples had low endo-(1,3)(1,4)-ß-D-glucanase activity.

## ACKNOWLEDGEMENTS

I would like to extend my gratitude to my major advisor, Dr. W. N. Eigel, for his guidance, suggestions and time during the research and writing of this thesis. Sincere thanks also are to my committe members - Dr. J. E. Marcy, Dr. C. R. Hackney and Dr. W. Barbeau for their assistance, especially in finalizing the thesis. Appreciation is expressed also to Yauching Jasinsky and Carolyn H. Harris for their technical guidance and help.

Last but not least, I would like to thank my parents, sisters and friends for the support and encouragement they gave me during my graduate studies.

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# CHAPTER 1

## INTRODUCTION

Traditional weaning foods are largely responsible for the high incidence of protein-energy malnutrition in African children (Boeh-Ocansey, 1989). Weaning involves the introduction of any solid, semi-solid or liquid food other than breast milk to infants weighing at least 5 kg or who are 4 - 6 months old (Brown, 1978). Weaning foods are usually based on local staples such as maize, sorghum, and millet. Typically, these foods are diluted so that they are low in nutrient content and caloric density.

The nutrients consumed and utilized by infants are influenced by food processing techniques, nutrient content as well as organoleptic characteristics of the food (Brown, 1991). One food processing technique that produces an increase in caloric density, nutrient availability and organoleptic properties of weaning foods is germination. Starch modification occurs during germination and produces products of low water holding capacity as well as increased flavor due to the production of mono- and disaccharides. A low water holding capacity is desirable since more dry solids can be used to prepare weaning foods without viscosity being a constricting factor. Thus the addition of more solids enhances caloric density of the meal.

Sorghum and cowpeas were chosen in this study due to their high availability in developing countries. In many countries, sorghum is used to prepare traditional alcoholic beverages, while cowpeas are used as accompaniments to meals. The prospect that sorghum will undergo germination and be utilized in the preparation of weaning foods, instead of brewing alcoholic beverages, is not popular in developing countries. However, the high incidence of malnutrition associated with traditional practices of feeding weanlings liquidized adult foods has led to the introduction of germination in the process. At 3 - 4 months, the digestive system of a weanling is incapable of absorbing nutrients from traditional foods (Brown, 1991).

Germination is part of the malting process. Malting consists of steeping, germination and drying or kilning. During germination, glycolytic enzymes are synthesized and hydrolyze starch into a variety of oligosaccharides and mono- and disaccharides. This process facilitates digestibility, reduces viscosity and elevates the caloric and nutrient content of foods (Desikachar, 1981; FNB, 1992; Marero et al, 1990).

After germination, seeds are dried to reduce moisture and prepare flour. Drying intervals reported in the literature have exceeded 10 hours in duration (Almeida-Dominguez, 1993; Hansen et al, 1989; Kulkarni et al, 1991; Malleshi et al, 1989; Marero et al, 1988a; Wang and Fields, 1978). Utilization of such lengthy drying times are of great concern for Third World Nations due to widespread scarcity of fuel resources. Africa has the lowest reserves of coal, oil and natural gas at 8.8%, 8.1%, and 6.1%, respectively of any continent (E.I.U., 1986). Prolonged drying times result in increased energy consumption and are less economical. Another disadvantage of prolonged dehydration is the possibility of inactivation of endogenous enzymes, which may result in lowered enzyme activity. Thus, shorter heat treatments would be more cost effective and may actually produce higher levels of enzyme activity. Higher enzyme activity is desirable to reduce viscosity during weaning food preparation. Therefore, nutrient and caloric content of weaning foods will increase due to the ability to produce foods with higher solids content.

Research has been conducted on $\alpha$-amylase, ß-amylase and endo-(1,3)(1,4)-ß-D-glucanase activity in germinated cereals. However, little information on the activity of ß-amylase and endo-(1,3)(1,4)-ß-D-glucanase in legumes is available. Only $\alpha$-amylase activity has been studied in flours used for preparation of weaning food. Therefore, the purpose of this study was to investigate the effect of variable drying and germination times on $\alpha$-amylase, ß-amylase and endo-(1,3)(1,4)-ß-D-glucanase activity in germinated sorghum

and cowpea flours.

# CHAPTER 2

# REVIEW OF LITERATURE

## *2.00 WEANING FOODS*

Weaning foods are important because adequate nutritional status is associated with sound defense mechanisms against infections. For example, according to the 1987 census, Tanzania had a mortality rate of 137 per 1,000 live births (Mosha and Svanberg, 1990). Malnutrition and disease were the main causes of death. A longitudinal study, conducted in Tanzania, used the anthropometric indicators of weight for age, weight for height and weight increment (Yambi et al, 1991). The study found a linear relationship between nutritional status and mortality among the 2,454 children (6 - 36 months) used in the project. The 37 who died underwent a decline in weight for age. Therefore, this finding indicates that mortality increases as nutrition status deteriorates.

The weaning period involves the supplementation of breast milk with other semi-solid or liquid foods. Weanlings fed solely on breast milk fall below the accepted reference weight for age. Poorly fed women in developing countries produce 500 - 600 ml of milk daily. For ideal growth, infants require more than 850 ml of breast milk daily (Brown, 1978).

Nutritional inadequacy during weaning period is ascribed to the following factors: food scarcity, inappropriate child-feeding patterns, and recurrent incidence of infections which may hinder nutrient intake and utilization (Brown, 1991). In much of the developing world, childhood malnutrition occurs in the same households where adults remain relatively well nourished. Such occurrences can be attributed to inequitable food distribution, characteristics of feeding behaviors, preparation or content of the diet or appetite of the child (Brown, 1991).

In developing countries weanlings are fed 3 times daily. During

mealtimes, only 2/3 of the food is usually consumed, due to limited gastric capacity, poor appetite, inappropriate composition or undesirable organoleptic properties of the diet (Brown, 1991). The gastric capacity per meal of weanlings is at least 40 g / kg of body weight, with an energy density of about 0.8 kcal / g, when a relatively dilute diet is consumed. In contrast, the observed mean energy density of weaning diets in Peru and Nigeria is about 0.5 kcal / g and less than 0.3 kcal / g, respectively (Brown, 1991).

Improvements in protein quality occur from blending cereals with legumes, to provide of lysine and sulfur amino acids, found to be limiting in cereals and legumes respectively, (Table 1)(Singh and Rachie, 1985). Cabezas et al (1982) reported a high protein efficiency ratio of 1.55 from complementing cowpeas with sorghum at an optimum ratio of 15 to 45 parts, respectively (Table 2). The protein quality of cowpea meal increases upon the addition of sulphur amino acids - cysteine and methionine (Table 3). Similar results have been reported by others and are characteristic of all food legumes (Sherwood et al, 1954 and Bressani, 1975).

### *2.00a GERMINATION AND WEANING FOODS*

Germination provides increases in digestibility, nutrient and caloric density, as well as reduced viscosity of starch based infant foods. Germination triggers the production of enzymes which hydrolyze starch into oligosaccharides. The advantages of germination as a process for producing nutrient dense weaning foods has been extensively studied.

Human milk has an energy density of 0.7 kcal / g, therefore a weaning diet should exceed 0.7 kcal / g and be at least 1 kcal / g. Traditional liquid gruels contain about 5% dry matter, and provide an energy density of 0.2 kcal / g (Mosha and Svanberg, 1990). The upper limit of 20 % dry matter, provides 0.7 - 0.8 kcal / g, and yields a highly viscous gruel. Mosha and Svanberg (1983) reported 2,500 ml of prepared gruel had to be consumed to meet a

Table 1. Essential Amino Acid Content (g/16g N) of Cowpeas and Sorghum Seeds ([A]Kochlar et al, 1988; [B]Almeida-Dominguez et al, 1993; [C]Rooney and Serna-Saldivar, 1991)

| Amino Acid | Cowpeas[A] | Sorghum[B] |
|---|---|---|
| Lysine[C] | 6.6 - 8.1 | 2.5 |
| Histidine | 2.9 - 4.7 | 2.4 |
| Arginine | 5.4 - 8.0 | - |
| Threonine | 3.6 - 4.5 | 3.6 |
| Valine | 4.9 - 5.7 | 6.0 |
| Isoleucine | 4.2 - 4.8 | 4.9 |
| Leucine | 7.6 - 8.5 | 15.9 |
| Tyrosine | 2.2 - 3.6 | 10.9 |
| Phenylalanine | 5.5 - 6.2 | |
| Methionine[C] | 1.5 - 2.3 | 3.4 |
| Cysteine | - | |

[C]FAO / WHO pattern g AA/100g protein: lysine = 5.44 and methionine = 3.52

Table 2. Complementary Effect of Cowpea Protein to Sorghum (Bressani, 1985)

| Diet (g) | | Weight Gain (g) | Protein Efficiency Ratio |
|---|---|---|---|
| Sorghum | Cowpea | | |
| 90.00 | 0 | 21 | 0.97 |
| 45.00 | 15.00 | 43 | 1.55 |
| 0 | 30.00 | 25 | 1.13 |

Table 3. Protein Quality and Sulphur Amino Acid Supplementation to Cowpea (Bressani, 1985)

B.V. Biological value

N.P.U. Net Protein Utilization

| Cowpea | B.V. (%) | N.P.U. (%) |
|---|---|---|
| Meal | 58.17 ± 2.31 | 50.60 ± 1.83 |
| Meal + Cystine | 80.25 ± 1.87 | 72.74 ± 0.94 |
| Meal + Cystine + Methionine | 94.61 ± 1.26 | 82.12 ± 1.07 |
| Meal + Methionine | 95.84 ± 1.45 | 81.46 ± 0.87 |
| Albumin | 101.72 ± 2.54 | 99.52 ± 1.85 |

child's daily requirement, when ungerminated sorghum flour was used. However, when germinated sorghum flour was used, the required volume dropped to 800 ml. Later in 1990, Mosha and Svanberg reported 3,540 ml of thin gruel, 870 ml of thick porridge and 710 - 870 ml of liquefied porridge were needed to meet a child's daily requirement. However, the actual intakes were 330 ml, 277 ml and 347 ml, respectively. Thus the number of meals per day needed to meet a child's daily requirement, decreased from 10.7 of the thin gruel to 2 - 2.5 of the liquefied diet.

According to Desikachar (1981), germination lowered the hot paste viscosity of a 10 % slurry more than heat processing operations such as flaking, puffing, parboiling and toasting. The viscosity of the unmalted rice, maize, wheat and green gram declined from a range of 1,000 cps - 4,000 cps to less than 100 cps after germination. Using wheat, Gopaldas et al (1988), prepared gruels of 15%, 20% and 25% dry matter and found the addition of malted cereal reduced viscosity by 30, 80 and 89%, respectively. A 90 - 92% reduction in viscosity occurred when malted cereal was added to the gruel after cooking, otherwise the viscosity was reduced by 40 - 57% (Gopaldas et al, 1988).

Cereal - legume formulations (70:30) of germinated rice-mungbean, rice-cowpea, corn-mungbean and corn-cowpea blends were studied for viscosity, acceptability and quality (Marero et al, 1988). The germination period for cereals and legumes was 72 and 48 hours, respectively. Germination reduced viscosity from 20,000 to 3,000 cps. Flavor and general acceptance were rated using a 9 point Hedonic scale, and averaged scores of 7 (like moderately). The products were stable for 6 months, microbially safe and well tolerated by infants.

A malted weaning food was prepared by blending malted sorghum and malted cowpea flours in the proportion of 70:30, respectively (Malleshi et al,

1989). The precooked weaning food was prepared by roller drying a cold water slurry consisting of 70% pearled sorghum flour and 30% toasted cowpea flour. Upon evaluation, the cooked paste viscosity of malted weaning food was lower than that of the roller dried food at all comparable slurry concentration. The protein content of the malted and roller dried formulations were 13.4 % and 13.0%, respectively. Available lysine in the malted sample was 3.85% and 2.95% in the roller dried sample. The protein efficiency ratio (PER) of malted sample was 2.26, and was significantly higher than the roller dried value of 1.87.

The reduction of antinutritional factors such as phytates, tannins, trypsin inhibitors, phytohemagglutinins and cyanogenic glycosides have been reported in germinated cereals-legumes formulations of rice, corn, mungbean and cowpeas (Marero et al., 1990). The study also found drying, dehusking, roasting and cooking significantly reduced antinutritional factors in the formulations. In vitro protein digestion increased, and no signs of stomach disorders, vomiting, rashes or fever occurred throughout the feeding trial in infants.

Almeida-Dominguez et al (1993), compared the effect of adding malted sorghum into a weaning food made of decorticated and press-dried pearl millet (70%) and cowpea (30%). Without the addition of malted sorghum flour, the gruel was highly viscous (1,007 cps). Addition of 5% malt reduced the viscosity of experimental and commercial weaning food pastes (20% dry matter) by more than 90% after 5 minutes of stirring at 30°C.

## *2.10 SORGHUM*

### a. Characteristics

Grain sorghum (*Sorghum bicolor L. Moench*) is characterized by drought resistance and adaptability to tropical conditions (Rooney and Serna-Saldivar, 1991). Cultivation of sorghum has been reported in India as early as 1 A.D.

As a domesticated crop, sorghum was introduced to Europe about 60 A.D. The first seeds were probably brought to the Western Hemisphere in slave ships from Africa (Bennett et al, 1990).

Sorghum belongs to the family *Graminae*, tribe *Andropogonae*. All annual sorghums belong to one species, *Sorghum vulgare*. A chief food grain in much of Africa and parts of Asia, sorghum is the world's third most utilized food grain, exceeded only by wheat and rice (Kramer, 1959). Sorghum is classified into four distinct groups: grain or non-saccharine sorghum used for human foods in the form of grits and flour; forage or saccharine sorghum (sorgo) used for forage, silage or molasses; broom corn and grass sorghum used for hay and pasture. Some of the local names for sorghum include Dwarf Yellow Milo, White Kafir, Red Kafir, Feterita, Hegari and Dorso (Bennett et al, 1990).

Sorghum remains dormant during drought and then resumes growth when rain comes. During drought, sorghum leaves roll as they wilt thus exposing less leaf surface area for transpiration. The abundant waxy covering on leaves and stalks and the large number of fibrous roots that extract moisture from the soil, also increase sorghum's drought resistance (Bennett et al, 1990).

**b. General composition**

Sorghum grain is composed of the pericarp (outer layer), endosperm (storage tissue) and germ (embryo). Sorghum cell walls contain about 4% pentosan, 28% ß-D-glucan and about 62% adhering protein (Palmer, 1992). Most sorghum pentosans are located in the pericarp. The carbohydrate content of the pentosans ranges from 68 to 85%. Glucose and arabinose are the carbohydrates found in larger quantities (Rooney and Serna-Saldivar, 1991). Crude fiber, present in the pericarp and endosperm cell walls, is composed of cellulose, hemicellulose and lignin.

The endosperm is a major storage tissue and is enclosed by the aleurone

layer (Rooney and Serna-Saldivar, 1991). Sorghum aleurone is comprised of a layer of cells, containing lipids and protein deposits (Palmer, 1992). Starch, the major component of sorghum, is found in the endosperm. Sorghum starch has granules of about 10 micrometers in diameter, and is composed of 70 - 80% branched amylopectin and 20 - 30% amylose (Palmer, 1992; Bennett et al, 1990). The germ is composed of the embryonic axis and the scutellum. The embryonic axis develops into a new plant, whereas the scutellum is a reserve tissue with large amounts of oil, protein, enzymes and minerals (Rooney and Serna-Saldivar, 1991).

**c. Changes during germination**

Germinated sorghum grains exhibit $\alpha$-amylase, carboxypeptidase, endo-ß-(1,3)-glucanase, pentosanase, limit dextrinase and endo-protease activity during malting. $\alpha$-Amylase is produced by the germinated embryo of sorghum (Palmer, 1992). The embryo initiates movement of food reserves by secreting large quantities of starch-degrading enzymes into the endosperm. In sorghum, endo-(1,3)(1,4)-ß-D-glucanase activity is low and the breakdown of endosperm cell walls is very limited. Cell walls appear to develop portals through which the starch degrading enzymes pass to mobilize starch and protein reserves of the endosperm (Palmer, 1992).

## *2.20 COWPEAS*

**a. Characteristics**

Cowpeas (*Vigna unguiculata L. Walpers*), also known as black-eye beans or southern peas, are a good source of protein, energy and other nutrients in developing countries (Uzogara and Ofuya, 1992). Thriving in regions of low relative humidity, cowpeas are grown extensively in 16 African countries. Nigeria and Niger produce 49.3% of the annual world crop (Singh and Rachie, 1985).

The word "legume" is derived from the Latin "legumen" meaning seeds

harvested in pods. An alternative term for edible seeds of leguminous plants is "pulses". Legumes with a small fat content are called "pulses", while those with a high fat content are identified as "leguminous oilseeds" (Salunkhe and Kadam, 1989).

In tropical Africa, cowpeas are interplanted with sorghum, millet and maize (Singh and Rachie, 1985). They are grown for fodder, ground cover or green manure. The ability of cowpeas to fix nitrogen efficiently, (up to 240 kg N/hectare and leave a fixed nitrogen deposit of up to 60-70 kg / hectare), improves soil fertility (Uzogara and Ofuya, 1992).

**b. General composition**

Mature legume seeds are composed of the seed coat (testa), the cotyledon and the embryo axis. At maturity, the endosperm is reduced to a thin layer surrounding the cotyledons. The endosperm is removed with the testa after soaking. Along with the testa, the hilum and the micropyle which make up part of the embryo axis, influence absorption and permeability of the seed coat to water. The inside surfaces of the seed coat and cotyledon have numerous hills and valleys which form interlocking structures. These structures may lead to difficulties in dehulling (Kadam et al, 1989). The carbohydrate content of cowpeas ranges between 30 to 50%, and over 50% of the starch is in the amylose form (Singh and Rachie, 1985).

**c. Changes during germination**

Carbohydrate digestibility is increased by germination. Ungerminated cowpea starch contains indigestible sugars such as stachyose, raffinose and verbascose, which have been shown to cause flatulence in individuals when consumed (Ndubuaku et al, 1989). These indigestible sugars belong to the raffinose family of oligosaccharides. The structural interrelationship of stachyose, raffinose and verbascose lies in the basic unit of sucrose and one or more $\alpha$-D-galactose units bound to glucose. These D-galactosyl groups are

found in nature joined to sugars such as D-glucose, sucrose and certain polysaccharides (Cristofaro et al, 1974). Cowpeas have an indigestible sugar content of 6 - 13%, while sorghum has 2.25% (Rooney and Serna-Saldivar, 1991).

Stachyose, raffinose and verbascose are unavailable for human nutrition due to the lack of specific degrading enzymes ($\alpha$-galactosidase and ß-fructosidase) in humans. These oligosaccharides are not hydrolyzed by gastrointestinal secretions, and thus pass to the colon where microbial fermentation occurs. Colonic microorganisms produce gas and free fatty acids, which can be accompanied by frequent belching, abdominal distension, diarrhoea and weakness (Ndubuaku et al, 1989). Akinlosotu and Akinyele (1991) reported germination increased and decreased monosaccharide and disaccharide contents of cowpeas, respectively. Monosaccharides can be utilized for energy by the growing child, and will also increase the palatability of cowpeas.

### *2.30 ENZYMES*

Enzymes are proteins that function as biological catalysts and are highly specific in reacting with one particular class of compounds such as proteins, carbohydrates or fats (deMan, 1990). As proteins, enzymes are susceptible to the same conditions that denature proteins such as heat, strong acids, strong bases, heavy metals and detergents (Whitaker, 1972). Enzymes react by forming enzyme substrate complexes in which the substrate is transformed into the desired product (deMan, 1990). A substrate is a compound which after combination with an enzyme is catalytically converted to a product (Whitaker, 1972).

$\alpha$-Amylase [EC 3.2.1.1], known as a liquefying and dextrinizing enzyme, possesses an endo-attack mechanism and hydrolyses $\alpha$-(1,4)-glucosidic linkages by-passing $\alpha$-1,6-glucosidic linkages. $\alpha$-Amylase rapidly decreases the viscosity

of starch solution (Robyt and Whelan, 1968). $\alpha$-Amylase is located in almost every fluid and tissue of living organisms.

ß-Amylase [EC 3.2.1.2] is an exo-enzyme, that removes maltose units from the non-reducing end of glucosidic chains. Hydrolysis stops at the branch points where $\alpha$-1,6-glucosidic links cannot be hydrolyzed by $\alpha$-amylase, resulting in limit dextrin formation (deMan, 1990). ß-Amylase is confined to seeds of higher plants, sweet potatoes and is present in limited amounts in oats, corn, rice, and sorghum. In mature seeds, ß-amylase is stored adjacent to the aleurone layer chemically attached to glutenin (Thoma et al, 1971).

Endo-(1,3)(1,4)-ß-D-glucanase [EC 3.2.1.6], hydrolyses ß-(1,4)-glucosidic linkages in ß-D-glucans containing 1,3 and 1,4 bonds (IUPAC and IUB, 1972). Endo-glucanase hydrolyzes lichenin and cereal ß-D-glucans containing 1,3 and 1,4 bonds (IUPAC & IUB, 1972). Prolonged hydrolysis by endo-(1,3)(1,4)-ß-D-glucanase yields primarily oligosaccharides of 3 to 4 glucose units.

### *2.40 MALTING*

Malting consists of three steps - steeping, germination and kilning or drying. During steeping, water is absorbed into the grain. Water absorption helps in the initiation of cell elongation, secretory activity of the embryo and activation of enzymes (Pyler and Thomas, 1991).

**a. Germination**

Germination is a physiological and biochemical process which involves the multiplication and enlargement of the acrospire and rootlet cells in a seed, to form a new plant (Witt, 1959). As germination begins, the aleurone layer secretes endo-(1,3)(1,4)-ß-glucanase which begins to digest endosperm cell walls. The secretion of endo-(1,3)(1,4)-ß-D-glucanase is important, since the digestion of the cell wall exposes the endosperm to the action of other enzymes secreted from the aleurone layer (Bamforth, 1982).

**b. Kilning**

Kilning follows germination in the malting process and involves drying "green malt" in an air oven 55 - 65°C (Owuama and Asheno, 1994). The purpose of kilning is to stop botanical growth and internal modification, prevent bacterial spoilage, reduce moisture for grain storage, partially or wholly destroy some hydrolytic and other enzymes and develop color and flavor compounds in the malt (Pyler and Thomas, 1991). These factors are affected by parameters such as the extent of starch modification, duration and levels of temperature - time sequence of the drying cycle, and moisture content of the grain at different stages of kilning (Owuama and Asheno, 1994).

Chemical changes associated with kilning occur in three phases; the germinative, enzymic and chemical phases. In the germinative phase, germination continues occurs for a limited time when moisture content is still high (40 to 50%), temperature is less than 50°C and there is access to uncontaminated air. During the enzymic phase, when moisture content is lower than 30%, some enzymic activity still continues as long as the temperature doesn't exceed 60°C. The enzymic phase helps in the production of amino acids and reducing sugars which combine to form colored substances.

Lastly, during the chemical phase colored products and aromatic substances are formed as a result of nonenzymatic browning or Maillard reaction (Bathgate, 1973). The browning reaction begins with the reaction of the amino group of amino acids or proteins with a glycosidic hydroxyl group of sugars. Eventually the reaction terminates with the formation of melanoidins (deMan, 1990).

The initial step of melanoidin formation involves the production of an N-substituted glycosylamine from an aldose or ketose reacting with a primary amino group of an amino acid or protein. The N-substituted glycosylamine then undergoes an Amadori rearrangement through the cation of the Schiff's Base, to yield an aldoseamine or ketoseamine (deMan, 1990). Two separate reaction

pathways occur, both of which lead to melanoidin formation. In the first pathway, 3-deoxyhexosones is formed, while the second pathway produces an $\alpha$-dicarbonyl intermediate. Both mechanisms undergo dehydration and yield $\alpha$, ß-unsaturated $\alpha$-dicarbonyl compounds which condense and polymerize to form soluble melanoidins (Type A). Further dehydration in the first pathway forms 5-hydroxy-methyl furfuraldehyde (HMF). HMF can also react via Schiff's Base to form melanoidins which, upon further polymerization, become insoluble. This second type of melanoidins (Type B) is responsible for the production of color, flavor and aroma compounds (Bathgate, 1973).

### *2.50 BREWING INDUSTRY AND MALT FLOUR*

Brewing utilizes the malting process to form reducing sugars which are a source of fermentable sugar in alcohol production. Since barley and sorghum are used in alcohol production, means of optimizing conditions for cereal utilization have been extensively studied. For example, Pathirana et al (1982), evaluated malted sorghum extract for reducing sugar content, diastatic and liquefying power. They reported a steeping time of 18 hours and a germination time of 4 - 5 days was optimum. Kilning at 100°C for 3 - 4 hours, was recommended due to rapid inactivation of hydrolytic enzymes at high temperatures. Morall et al (1986), germinated sorghum for 0.5 to 6 days over a temperature range of 24 - 36°C and under variable moisture conditions to study their effect on malt quality and malting loss. Germination time, temperature and moisture all had significant effect on malt diastatic power ($P<0.001$).

In weaning foods, malted flour enhances nutrient and energy availabilty and improves organoleptic attributes. Thus, glycolytic enzymes are the key to viscosity reduction in weaning gruels. Therefore, it is important to minimize the loss of such enzymes due to prolonged heat exposure during kilning. Information on how the exposure to different drying and germination times

affect the behavior of $\alpha$-amylase, ß-amylase and endo-(1,3)(1,4)-ß-D-glucanase activity in sorghum and cowpeas is scarce. Thus the objectives of the study were to determine:-

(a) The effect of germination time of 0, 1, 2 and 3 days on levels of $\alpha$-amylase, ß-amylase and endo-(1,3)(1,4)-ß-D-glucanase activity in sorghum and cowpeas; and

(b) The effect of drying times of 2.5, 5, 7.5 and 10 hours on $\alpha$-amylase, ß-amylase and endo-(1,3)(1,4)-ß-D-glucanase activity in sorghum and cowpeas germinated for 0, 1, 2 and 3 days.

# CHAPTER 3

# MATERIALS AND METHODS

## *3.00 MATERIALS*

Corn-starch, barley-ß-glucan, *p*-nitrophenyl-maltopentaose (PNPG-5), yeast *α*-glucosidase [E.C. 3.2.1.20] Type 1, *α*-amylase Type II [E.C.3.2.1.1] from *Bacillus* species and sweet potato ß-amylase [E.C.3.2.1.2] Type I-B were from Sigma Chemical Co., St. Louis, Missouri. Grain sorghum was supplied by Dekalb Plant Genetics (Robstown, Texas) and cowpeas were purchased from Kroger Co., Blacksburg, Virginia.

## *3.10 SUBSTRATE PREPARATION*

### a. Corn-starch ß-limit dextrin

Corn-starch ß-limit dextrin was made following standard American Association of Cereal Chemists (AACC) Method 22-01 (1983). Corn-starch (10 g) was suspended in distilled water (25 ml) and the solution was added to boiling water (300 ml). The mixture was boiled for 2 minutes, and cooled to room temperature with stirring. After the addition of 25 ml buffer (4 *M* sodium acetate, pH 4.7), ß-amylase (10 $\mu$l) and toluene (40 $\mu$l), the sample was mixed for 1 minute. The final volume was increased to 500 ml with distilled water. The solution was then incubated at room temperature (25°C) in a shaking water bath for 20 hours.

After incubation, the corn-starch solution was heated to just boiling and cooled rapidly in a cold water bath to denature the ß-amylase. The solution was dialyzed for 48 hours against distilled water at 4°C for 2 days (Marchylo and Kruger, 1978). Dialysis was performed in Spectra/Por molecular-porous membrane tubings (Spectrum Medical Industries, Inc., Houston, Texas), with a molecular weight cut off of 1000 Daltons. The tubes were placed in 4 L buckets filled with cold distilled water and refrigerated. Water was changed

twice daily.

After dialysis, the cornstarch samples were dispensed into LabConco freeze-drying flasks and shell frozen in an acetone / dry-ice bath. The samples were freeze-dried overnight using a Virtis Sentry Freezemobile 12SI (Gardiner, New York). Following lyophilization, samples were stored in plastic Ziplock™ bags in stoppered glass bottles at 4°C.

1. Reduced ß-limit dextrin stock solution

Corn-starch ß-limit dextrin (2 g) was dissolved in boiling distilled water (77.5 ml) and cooled to room temperature in an ice-bath. Sodium borohydride (0.74 *M*) was dissolved in distilled water (2.5 ml) and added dropwise with stirring. The mixture was stirred for 30 min and cooled for 1 min in an ice-bath at 5 min intervals. The solution was allowed to stand at room temperature for 2 hrs and refrigerated at 4°C prior to use (Kruger and Marchylo, 1972).

2. 0.5% Reduced ß-limit dextrin working solution

Acetone (0.3 ml) was stirred in 15 ml of the stock solution and the pH adjusted to 5.5 with 1 *N* acetic acid. The volume was adjusted to 75 ml with 0.05 *M* sodium acetate buffer, pH 5.5 (Kruger and Marchylo, 1972). The working solution was the substrate used for the analysis of *α*-amylase activity.

**b. 0.25% Barley-ß-glucan**

Barley ß-glucan (0.05 g) was dissolved in 20 ml sodium phosphate buffer (50 m*M*, pH 6.5) (McCleary, 1986). The solution was heated to aid dissolution, cooled to room temperature and stored at 4°C. ß-Glucan (0.25%) was used to analyze for endo-(1,3)(1,4)-ß-D-glucanase activity.

**c. PNPG-5 Substrate**

The substrate for determining ß-amylase activity contained PNPG-5 (5 m*M*) and *α*-glucosidase (100 U) per 1 ml distilled water (McCleary and Codd, 1989).

### *3.20 BUFFER AND REAGENT PREPARATION*

**a. Sodium maleate buffer**

Sodium maleate buffer (50 mM, pH 5.2) contained maleic acid (50 m*M*), calcium chloride (2 m*M*), sodium chloride (50 m*M*) and sodium azide (3 m*M*) (Corder and Henry, 1989). Solutes were dissolved in distilled water.

**b. Total ß-amylase extraction and dilution buffers**

Buffer A (pH 6.2) was made of maleic acid (100 m*M*); disodium EDTA (1 m*M*); bovine serum albumin (BSA, 1 mg/ml) and sodium azide (3 m*M*). Buffer B (pH 6.2) was similar to Buffer A, but contained cysteine (20 m*M*) (McCleary and Codd, 1989). Buffer A was used for enzyme dilution and in assay mixtures, while Buffer B was used to extract total ß-amylase.

**c. Dinitrosalicyclic acid (DNS)**

DNS was prepared by dispensing 10 g NaOH, 10 g DNS, 2 g phenol and 200 g Rochelle salt (sodium potassium tartrate) in 500 ml distilled water. After dissolving, the solution was diluted to 1 L with distilled water and refrigerated at 4°C in a brown bottle (Stauffer, 1989). Before use, 0.05% $Na_2SO_3$ was added to the volume of reagent used.

### *3.30 GERMINATION*

In separate beakers, sorghum and cowpea seeds (100 g) were sanitized in 1 % sodium hypochlorite solution (150 ml) for 20 min at room temperature (Figure 1) (Aisien et al, 1983). After sterilization, the seeds were steeped separately in distilled water (400 ml) for 6 hrs at room temperature (Marero et al, 1988a).

The seeds were spread evenly on trays lined with a single layer of moist filter paper (Aisien et al, 1983). The trays were then covered loosely with Saran™ wrap and stored at 25°C for 0, 24, 48 or 72 hrs to allow germination to occur. The seeds were rinsed every 8 hours with distilled water (Marero et al, 1988a).

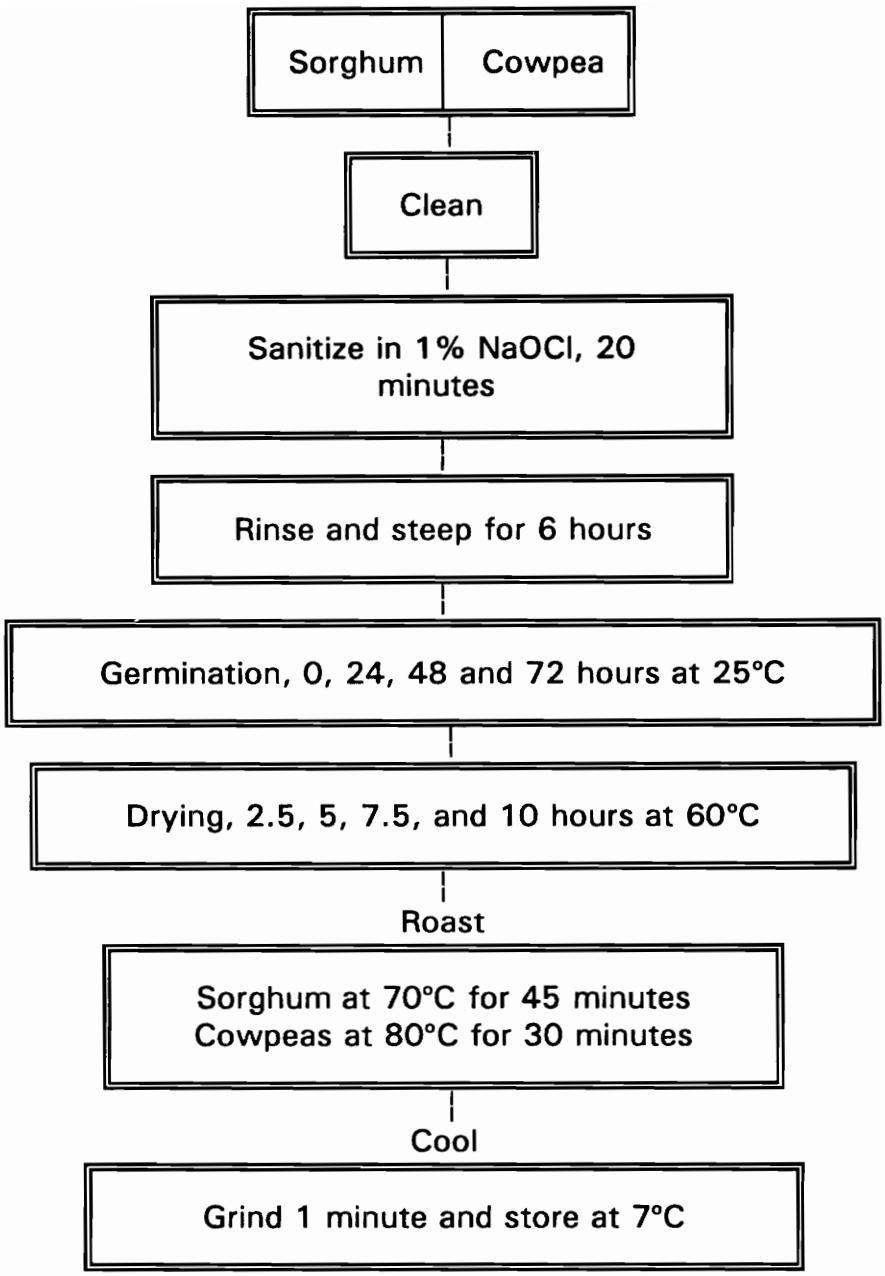


Figure 1. Flour preparation from germinated sorghum and cowpeas

After germination times of 0, 24, 48 or 72 hrs, grains were dried in a Blue-M Constant Temperature Cabinet air-oven (Blue Island, IL) at 60°C for 2.5, 5, 7.5 or 10 hrs. Sorghum was then toasted in a 70°C oven for 45 min, and cowpeas for 30 min at 80°C (Malleshi et al, 1989). The toasted seeds were cooled for 10 - 15 min and ground separately in a Regal™ coffee grinder for 1 min. Ground samples were stored desiccated in labelled double Ziplock™ bags, at 7°C. Complete replicates of each germination and drying time were done.

### *3.40 PROXIMATE ANALYSIS*

Moisture, protein, fat and ash content of raw grains were determined following standard AACC (1983) procedures. Results were expressed on a dry-weight basis.

**a. Moisture determination**

Sorghum and cowpea samples (2 g), were placed in separate aluminum dishes and dried in a 135°C air oven (Freas Precision Scientific Air Oven Model 625, Chicago, IL) for 2 hours (AACC Method 44-19, 1983).

The samples were then immediately placed in a desiccator and cooled to room temperature for 45 minutes. The new weight was noted and % moisture loss was determined by the following formula:

$$\% \text{ moisture loss} = \frac{\text{Loss of moisture}}{\text{Original sample weight}} \times 100$$

**b. Protein determination**

Crude protein (N X 6.25%) was determined using the Kjeldahl Nitrogen Procedure (AACC Method 46-12, 1983). Ground samples (0.5 g) were added into digestion tubes containing 4 g catalyst (1 : 50 copper sulfate - sodium sulfate ratio) and concentrated $H_2SO_4$ (15 ml). The digestion tubes were placed onto a Buchi 430 Digestor (Brinkmann Instruments, Inc., Westbury, NY) until samples turned green and then cooled for 30 min. During digestion,

nitrogenous compounds are converted to ammonium sulfate.

After cooling, samples were distilled in an automated Buchi 322 Distillation Unit (Brinkmann Instruments, Inc., Westbury, NY) connected to a printer. After distillation, ammonia is estimated by titration using 0.1 *N* HCL. The instrument was set to give % nitrogen. Thus % protein was calculated using the following formula:

$$\%\ Protein = (\%\ Nitrogen) \times 6.25$$

The value (6.25) is a conversion factor (CF) for calculating protein content in sorghum and cowpeas (Almeida-Dominguez et al, 1993).

**c. Fat determination**

Fat content was determined by a Soxtec System HT 1043 Extraction Unit (Tecator, Inc., Herndon, Virginia). Pre-dried samples (2 g) were placed into pre-weighed thimbles covered with cotton plug (AACC Method 30-25, 1983). The thimbles were inserted into the condensers turned to extraction mode and then moved to boiling position. Diethyl ether (25 - 50 ml) was added into tared extraction cups with boiling chips and then inserted underneath the thimbles. The extraction mode knobs were moved to "boiling" position, causing the thimbles to be immersed in the solvent.

The samples were boiled and rinsed; solvent was collected in the condenser for 15 min. Samples were then evaporated for 15 min, and extraction cups were weighed. Fat content was calculated using the following formula:

$$\%\ Crude\ fat = \frac{Weight\ of\ fat}{Sample\ weight} \times 100$$

**d. Ash determination**

Samples (5 g) were aliquoted into weighed porcelain crucibles and placed

into a Fisher Isotemp Model 495 Ashing Furnace (Chicago, Illinois). The muffle furnace was programmed following manufacturer's instructions (Fisher Scientific Company, Chicago, Illinois) and AACC's Method 08-01, 1983. The furnace temperature was set to increase at 5°C per min to attain a transition temperature of 250°C. After holding for 90 min, the temperature increased at a rate of 5°C per min to reach a final temperature of 550°C. Samples were held at 550°C for 10 hrs. After samples had cooled, they were reweighed. Ash content was calculated using the following formula:

$$\% \ Ash = \frac{Weight\ of\ residue}{Sample\ weight} \times 100$$

**e. Carbohydrate determination**

Carbohydrate content (CHO) was determined by difference.

%CHO = 100 - (%Protein + %Moisture + %Fat + %Ash)

### *3.50 ENZYME EXTRACTION*

**a. α-Amylase and endo-(1,3)(1,4)-ß-D-glucanase extraction**

Sorghum or cowpea flour (0.8 g) was vortexed with 8 ml sodium maleate buffer (50 m*M*, pH 5.2). Samples were centrifuged in a Dynac centrifuge (Persipanny, NJ) at 1000X g for 10 min (Corder and Henry, 1989). The extract was immediately decanted into new labelled test tubes and was assayed for reducing sugar equivalents, α-amylase and endo-(1,3)(1,4)-ß-D-glucanase.

**b. ß-Amylase extraction**

Flour (sorghum or cowpea) samples (0.5 g) were weighed into 15 ml capacity Corex centrifuge test tubes with 5 ml Buffer B (pH 6.2). The tubes were vortexed and allowed to incubate at room temperature for 2 hrs. After centrifugation at 1000X g for 10 min, the supernatant (0.2 ml) was diluted to 10 ml with Buffer A (pH 6.2). An aliquot (0.2 ml) was further diluted to 2 ml in Buffer A and incubated at 40°C for 10 min prior to assay (McCleary and

Codd, 1989).

**c. Action of DNS with enzyme extracts**

Germination triggers endogenous enzyme activity, which results in starch hydrolysis and the production of oligosaccharides. Thus, extracts will contribute to background colorimetric reaction with DNS. Enzyme extracts were reacted with DNS and the resultant absorbance value was regarded as a blank. The blank was substracted from corresponding absorbance values of enzyme extracts incubated with either limit dextrin or ß-glucan substrates. Units of enzyme activity were then determined from maltose and glucose standard curves, respectively.

Extracts (1 ml) were placed into screw capped test tubes in triplicate. Distilled water (1 ml) and DNS (3 ml) were added and samples were placed in a boiling water bath for 5 min. After cooling in an ice bath, distilled water (6 ml) was added to each tube. Absorbance was read at 540 nm in a Perkin Elmer Lambda 3 UV/VIS Spectrophotometer (Oak Brook, IL).

## *3.60 ENZYME ASSAYS*

**a. Calculation of enzyme activity**

Activity of crude enzyme extracts from malted sorghum and cowpea flours was expressed into "units of activity" at 25°C (77°F) using the following formulae:

$$RS(mg/ml) = \frac{\Delta_{540nm} - Constant}{X\text{-}Coefficient}$$

$$Units\ of\ activity\ (\mu moles/min/ml) = \frac{RS \times M.W.}{10} \times 10^6$$

where:-

Reducing Sugar (RS) = Maltose or glucose concentration (mg/ml)

$A_{540nm}$ = $A_{540nm}$(Reaction) - $A_{540nm}$(Blank)

Constant and X-Coefficient values obtained from regression output of standard curves.

M.W. = Molecular weight of glucose or maltose

10 = Incubation time in minutes

**b. *α*-Amylase assay**

Enzyme extract (1 ml) was incubated for 10 min at 25°C, with 0.5% reduced ß-limit dextrin working solution (1 ml ). DNS (3 ml) was added, tubes were capped and placed in boiling water bath for 5 min. Upon cooling, distilled water (6 ml) was added and tubes were vortexed. Absorbance was read at 540 nm. One unit of enzyme activity is defined as the amount of enzyme which releases 1$\mu$mole of maltose reducing sugar equivalents/ml/min under defined assay conditions.

**c. Endo-(1,3)(1,4)-ß-D-glucanase assay**

ß-D-Glucanase activity was determined using the same assay as described above for *α*-amylase, except 0.25% B-glucan was used as substrate and sodium phosphate (50m*M*, pH 6.5) served as a buffer (McCleary, 1986). One unit of enzyme activity is defined as the amount of enzyme which releases 1 $\mu$mole of glucose reducing sugar equivalents/ml/min. The formula to calculate enzyme activity was identical to that used for *α*-amylase assay.

**d. ß-Amylase assay**

Enzyme extract (0.2 ml) was incubated with pre-equilibrated PNPG-5 substrate mixture (0.2 ml), for 10 min in a 40°C water bath. The reaction was terminated by addition of 1% Trizma base (3 ml, pH > 10). Color development was monitored by absorbance at 410 nm. One unit of enzyme activity is defined as the amount of enzyme which releases 1 $\mu$mole of *p*-nitrophenol/min

under defined assay conditions. ß-Amylase activity was determined by the formula of McCleary and Codd (1989).

### *3.70 EXPERIMENTAL DESIGN*

The statistical method of 2 by 2 by 4 completely randomized factorial design was used with 2 completer replications. This design consisted of two samples (sorghum and cowpeas seeds) with two parameters (germination and drying time). Each parameter was studied at 4 different levels.

Two - way analysis of variance (ANOVA) was carried out using the SAS General Linear Model (GLM) procedure (1990, SAS Institute Inc., Cary, NC). Differences amongst means were determined using the Least Squares Means (LSM) procedure. Means were computed for the significant sources of variation using Duncan's Multiple Range Test ($\alpha = 0.05$). Analysis was used to answer the following questions:-

(a) Does drying affect enzyme activity in malted sorghum and cowpea flours?

(b) Does germination affect enzyme activity in malted sorghum and cowpea flours?

(c) Does the combination of (a) and (b) affect enzyme activity in both sorghum and cowpeas?

(d) At what drying and germination times does optimum enzyme activity occur in sorghum and cowpea flours?

# CHAPTER 4

# RESULTS AND DISCUSSION

## *4.00 PROXIMATE ANALYSIS*

Proximate analysis of ungerminated grains showed cowpeas had a mean protein and ash content of 20.59% and 3.22% respectively. Ungerminated sorghum contained 8.47% protein and 1.17% ash (Table 4). Even though the protein content in cowpeas is very high, the amount that can be utilized by the human body is low. The low net protein value (NPV) of 9.4% reported for cowpeas is due to low amounts of sulphur containing amino acids (Boeh-Ocansey, 1989). The low protein content in sorghum (Table 4) is due to the limitting amino acid, lysine (Boeh-Ocansey, 1989). Thus the blending of cereals with legumes will serve to provide the respective limiting amino acids and improve overall protein quality of the product.

Cowpea had a lower carbohydrate content (57.22%) than sorghum (69.53%). The carbohydrate content of cowpea and sorghum can be attributed to factors such as the amount of protein, starch and oligosaccharides in the samples. The nutrient values obtained from the samples were slightly lower than those of Bressani (1985) and Almeida-Dominguez et al (1993) (Table 4). Factors causing such a variation could arise from location in which the crops were grown and harvested, as well as seed variation.

## *4.10 OPTIMUM CONDITIONS FOR DETERMINATION OF ENZYMES*

Preliminary experiments were conducted to find optimum conditions for $\alpha$-amylase, ß-amylase, endo-(1,3)(1,4)-ß-D-glucanase activity. These experiments were conducted to determine the effect of incubation time and type of substrate on each enzyme. Results were used to determine optimum conditions for each enzyme and to demonstrate that each substrate was hydrolyzed only by one particular enzyme. The substrates used were corn-starch ß-limit-dextrin, *p*-nitrophenyl maltopentaose (PNPG-5) and barley-ß

Table 4. Comparison of nutrients in samples to those reported in literature (Bressani, 1985[C]; and Almeida-Dominguez et al, 1993[D]). Values expressed on dry weight basis.

| Nutrient (%)[A] | Cowpea (E) | Cowpea[C] | Sorghum (E) | Sorghum[D] |
|---|---|---|---|---|
| Moisture | 9.59 ± 0.08 | N.D. | 9.73 ± 0.06 | N.D. |
| Protein | 20.59 ± 0.55 | 24.80 | 8.47 ± 0.23 | 10.50 |
| Carbohydrate | 57.22 ± 0.63 | 63.60 | 69.53 ± 0.30 | 79.10 |
| Fat | 0.71 ± 0.03 | 1.90 | 2.32 ± 0.06 | 3.40 |
| Ash | 3.22 ± 0.002 | 3.60 | 1.17 ± 0.08 | 1.60 |
| Crude Fiber | N.D. | 6.30 | N.D. | N.D. |

[A] Mean of 3 observations ± Standard Error (SE)
E = Ungerminated experimental samples

glucan.

Conditions for ß-amylase determination were according to McCleary and Codd (1989), who recommended a 10 min incubation of PNPG-5 with enzyme extracts. Preliminary experiments demonsrated that PNPG-5 was not degraded by exogenous *α*-amylase nor endo-(1,3)(1,4)-ß-D-glucanase. PNPG-5 was rapidly hydrolyzed by exogenous ß-amylase (0.506 *u*mole / ml/ min) to yield maltose and *p*-nitrophenyl maltotriose (PNPG-3).

The substrate (PNPG-5) also contains *α*-glucosidase and cysteine. When the mixture is incubated with ß-amylase, maltose is released and allows *α*-glucosidase to rapidly cleave the other product (PNPG-3) to glucose and *p*-nitrophenol. The release of *p*-nitrophenol is used to monitor the activity of ß-amylase, since *p*-nitrophenol forms a yellow color with Trizma base. *p*-Nitrophenol concentration was determined from a standard curve. PNPG-5 is resistant to *α*-glucosidase hydrolysis. Hydrolysis of PNP-maltosaccharides by *α*-amylase is inhibited by the D-gluco-oligosaccharide chain length of 5. PNPG-5 cannot be used to measure *α*-amylase activity since this enzyme requires (1,4)-*α*-linked maltosaccharides with a minimum chain length of 7 to 9 D-glucosyl residues (McCleary and Codd, 1989).

Corn-starch ß-limit-dextrin could not be degraded by either ß-amylase or by endo-(1,3)(1,4)-ß-D-glucanase at incubation intervals of 0, 10, 20, and 30 min. ß-Limit dextrin was formed from excess treatment of starch with ß-amylase, which hydrolyzes the amylose to produce maltose and degrades amylopectin from the non-reducing ends to the *α*-(1,6)-branch points. Therefore, the resultant substrate cannot be further degraded by ß-amylase. However, *α*-amylase is capable of hydrolyzing ß-limit dextrin to produce oligosaccharides of 2 to 6 glucose units. Thus, when used to detect amylase activity, any further reaction of ß-limit dextrin will be due to the presence of *α*-amylase. Endo-(1,3)(1,4)-ß-D-glucanase cannot hydrolyze ß-limit dextrin, as it

is made of starch which is found in the endosperm of seeds. The location of substrate material for endo-(1,3)(1,4)-ß-D-glucanase is located in the seed cell walls.

Hydrolysis of barley-ß-glucan by exogenous endo-(1,3)(1,4)-ß-D-glucanase produced values of 42.21, 49.80 and 88.85 μmoles glucose/ml/min at 5, 10 and 20 min incubation, respectively. Therefore an incubation time of 10 min was chosen to allow the expression of endo-glucanase activity. Corresponding values for exogenous action of α-amylase were 0, 0.13 and 15.42 υmoles glucose/ml/min of glucose, indicating the presence of small amounts of starch in the barley-ß-glucan substrate. ß-Glucan, an unbranched polysaccharide made of 70% 1,4-linked and 30% 1,3-linked ß-D-glucopyranosyl units, cannot be hydrolyzed by either α or ß-amylase. The major products of endo-(1,3)(1,4)-ß-glucanase hydrolysis are 3-O-ß-cellobiosyl-D-glucose and 3-O-ß-cellotriosyl-D-glucose (Wood et al, 1989).

The hydrolysis of glycosides such as ß-limit dextrin is accompanied by an increase in the number of reducing groups. Thus, with appropriate chemical reaction a colored compound can be formed which is directly related to the concentration of reducing sugar in the mixture. Dinitrosalicyclic acid (DNS) was the reagent used. DNS measures the reducing hemiacetal groups in starch fragment, resulting from hydrolysis by enzyme extracts. DNS forms nitroaminosalicyclic acid, which when measured with a spectrophotometer, corresponds to the reducing sugar concentration formed (Asp, 1990).

### *4.20 STEEPING*

Samples were sanitized in 1% sodium hypochlorite solution to minimize mold growth. The seeds were washed to remove nonviable cells and thus decrease the occurrence of unpleasant odors. The primary purpose of steeping is to introduce water into the kernel to permit germination and produce a well modified malt. Marero et al (1988a) reported steeping legumes for longer

periods of up to 16 hours resulted in the production of foul smell indicative of deterioration. Gopaldas et al (1988) observed spoilage indicated by slimy water and starch being leached from the grain when steeping wheat for more than 12 hrs. Based on these findings, 6 hrs of steeping were chosen for this project.

### *4.30 ENZYME ACTIVITY*

#### a. β-Amylase activity

During this investigation, neither soluble nor insoluble ß-amylase activity could be detected in either ungerminated or germinated sorghum and cowpea samples. ß-amylase is not as prevalent as α-amylase and only low levels have been reported in sorghum and ungerminated cereals. In cereal flours, ß-amylase occurs in both soluble and insoluble forms (Thoma et al, 1971). Insolubility could be due to intermolecular disulfide bonding between the enzyme and other insoluble proteins (Whitaker, 1972). The inability to detect ß-amylase could arise from factors such as reverse inhibition and from the action of endogenous proteases. Reverse inhibition seems most probable due to high turnover of reducuing sugars produced by α-amylase, which was very active during the germination period.

In sorghum, low ß-amylase activity has been attributed to the interaction of ß-amylase with polyphenols during aqueous extraction to form insoluble polyphenol enzyme complexes (Ratnavathi and Ravi, 1991). Research on ß-amylase in legumes is scanty.

#### b. Germination and enzyme activity

##### 1. α-Amylase

Germination had a highly significant effect on both cowpea and sorghum α-amylase activity ($P<0.05$). For both samples, α-amylase activity increased with longer germination time (Figures 2). Malleshi et al (1989) reported similar behavior. They found α-amylase activity had attained its optimum at 3 days germination and began to decline at 4 days. The effect of

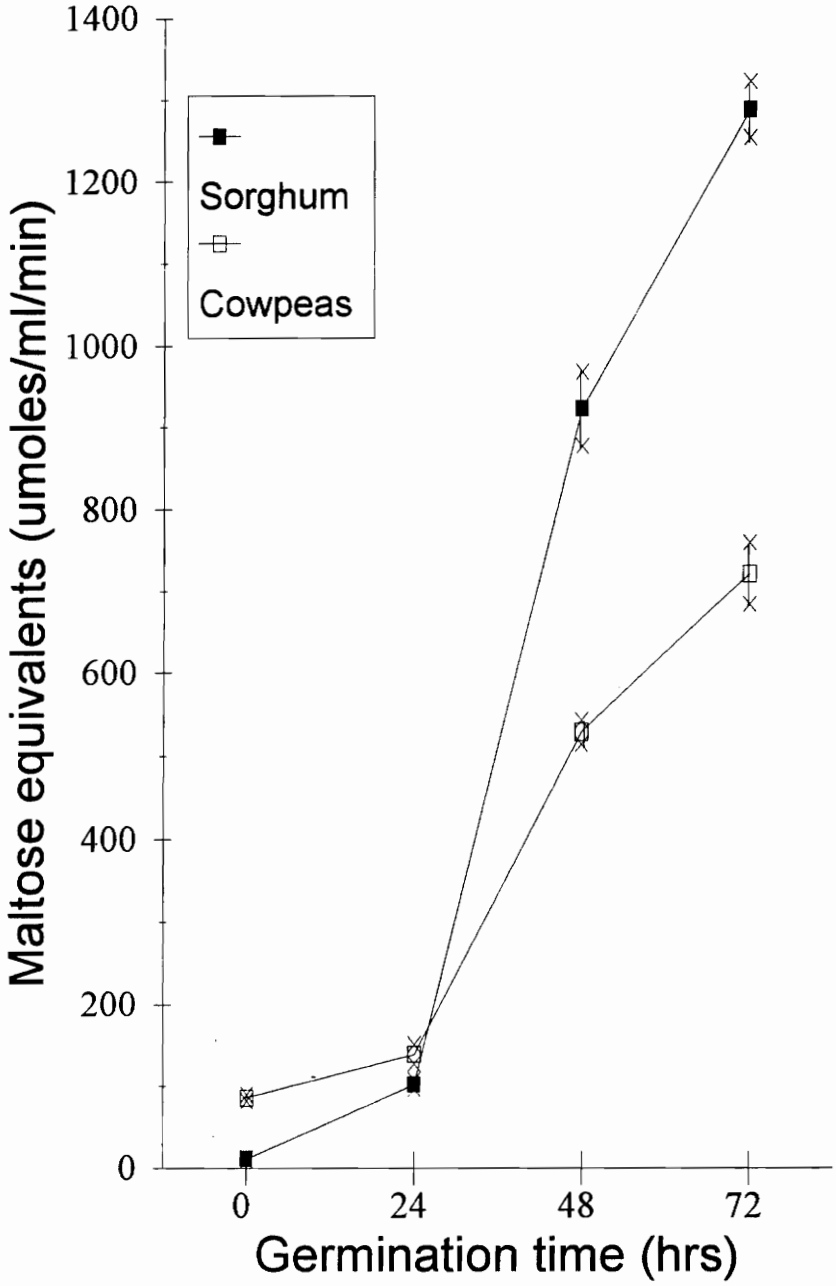


Fig. 2. Germination effect on alpha-amylase activity

germination and drying time on $\alpha$-amylase activity was significant (P = 0.0001) in both cowpeas and sorghum $\alpha$-amylase activity (Table 5 and 6). However, cowpea $\alpha$-amylase activity was not significant at 0 and 24 hrs of germination (Table 7).

**2. Endo-(1.3)(1,4)-ß-D-glucanase**

Analysis of variance indicated endo-(1,3)(1,4)-ß-D-glucanase was significantly increased by germination ($P<0.05$) in sorghum, but not in cowpeas ($P<0.05$) (Tables 7 and 8). Endo-(1,3)(1,4)-ß-D-glucanase activity was negligible in cowpeas (Figure 3), due to the endosperm being reduced to a thin layer surrounding the cotyledons upon maturity. Another reason for low endo-(1,3)(1,4)-ß-D-glucanase activity in cowpeas, is caused by the removal of endosperm with the testa upon soaking. Sorghum endo-(1,3)(1,4)-ß-D-glucanase was significantly different ($P<0.05$) at 2 days of germination (Table 8), while in cowpeas significance occured at 3 days (Table 7). Compared to sorghum $\alpha$-amylase activity (Table 8), the activity of sorghum endo-(1,3)(1,4)-ß-D-glucanase is low. Research has shown that endo-(1,3)(1,4)-ß-D-glucanase activity is very low in sorghum (EtokAkpan, 1992 and Palmer, 1992).

Cell wall of sorghum contains about 30% (w/w) ß-D-glucan, 5% (w/w) pentosan and 60% (w/w) protein. During germination only limited degradation of ß-glucan had been reported so that hydrolysis of cell wall pentosan is limited. Studies of cell wall breakdown in sorghum have shown that the starch content of endosperm cells is reduced during germination, despite the presence of undegraded cell walls (Etokakpan and Palmer, 1990; Palmer, 1991; and Etokakpan, 1992). Such behavior is believed to be caused by the formation of portals in endosperm cell walls of sorghum during malting. These portals have been observed by Scanning Electron Microscopy in the endosperm cell wall. The portals apparently allow amylolytic and proteolytic enzymes to enter the endosperm and hydrolyze starch and protein reserves (Palmer, 1991). Further

Table 5. Effect of germination on cowpea enzyme activity[x] (*u*moles reducing sugar/ml/min)

| Germination (hrs) at 25°C | *α*-amylase | Glucanase |
|---|---|---|
| 0 | $85.57^{b} \pm 3.65$ | $4.54^{b} \pm 1.07$ |
| 24 | $138.25^{c} \pm 11.63$ | $9.26^{b} \pm 2.13$ |
| 48 | $528.28^{b} \pm 13.74$ | $16.32^{b} \pm 2.12$ |
| 72 | $720.89^{a} \pm 37.75$ | $62.47^{a} \pm 6.51$ |
| P-value | 0.0001 | 0.0001 |
| F-value | 244.60 | 27.92 |

[x]Dry weight values and means of 48 observations ± SE

[a,b,c] Means in same column with different letters are significantly different at $P < 0.05$.

Table 6. Effect of drying and germination sorghum $\alpha$-amylase activity[X](umoles maltose/ml/min)

| Drying time (hrs) | G = 0 | G = 24 | G = 48 | G = 72 |
|---|---|---|---|---|
| 2.5 | 4.57 ± 1.85 | 68.88 ± 2.50 | 711.91 ± 32.47 | 1191.32 ± 59.26 |
| 5 | 18.56 ± 0.67 | 139.83 ± 9.67 | 905.73 ± 100.12 | 1341.48 ± 45.18 |
| 7.5 | 15.17 ± 1.55 | 83.38 ± 12.32 | 917.45 ± 98.44 | 1425.10 ± 50.81 |
| 10 | 5.88 ± 1.17 | 114.93 ± 5.13 | 1157.65 ± 50.46 | 1196.51 ± 78.26 |
| P-value | 0.0001 | | | |
| F-value | 4.36 | | | |

[A]Values are means of 24 observations ± SE

G = Germination (hrs)

Table 7. Effect of drying and germination on cowpea $\alpha$-amylase activity[A] ($\mu$moles maltose/ml/min)

| Drying time (hrs) | G = 0 | G = 24 | G = 48 | G = 72 |
|---|---|---|---|---|
| 2.5 | 78.59 ± 5.98 | 129.56 ± 10.95 | 472.96 ± 6.91 | 509.22 ± 56.41 |
| 5 | 99.48 ± 10.01 | 216.88 ± 32.04 | 522.8016 ± 6.49 | 819.88 ± 86.68 |
| 7.5 | 76.41 ± 3.32 | 98.18 ± 4.40 | 632.95 ± 25.74 | 728.07 ± 7.61 |
| 10 | 87.80 ± 2.39 | 108.39 ± 8.79 | 484.40 ± 13.55 | 826.36 ± 37.26 |
| P-value | 0.0001 | | | |
| F-value | 4.39 | | | |

[A]Values are means of 24 observations ± SE

Table 8. Germination effect on sorghum enzyme activity[x] (*u*moles reducing sugar/ml/min).

| Germination (hrs) at 25°C | *α*-Amylase | Glucanase |
|---|---|---|
| 0 | 11.04133[d] ± 1.04 | 7.8298[c] ± 1.34 |
| 24 | 101.7643[c] ± 5.59 | 37.8067[b] ± 5.17 |
| 48 | 923.1825[b] ± 45.34 | 90.4589[a] ± 13.08 |
| 72 | 1288.61[a] ± 33.96 | 20.31[bc] ± 15.40 |
| P-Value | 0.0001 | 0.0001 |
| F-value | 603.64 | 29.01 |

[x]Dry weight values and means of 48 observations ± SE

[a,b,c] Means followed by different letters in the same column are significantly different at $P < 0.05$

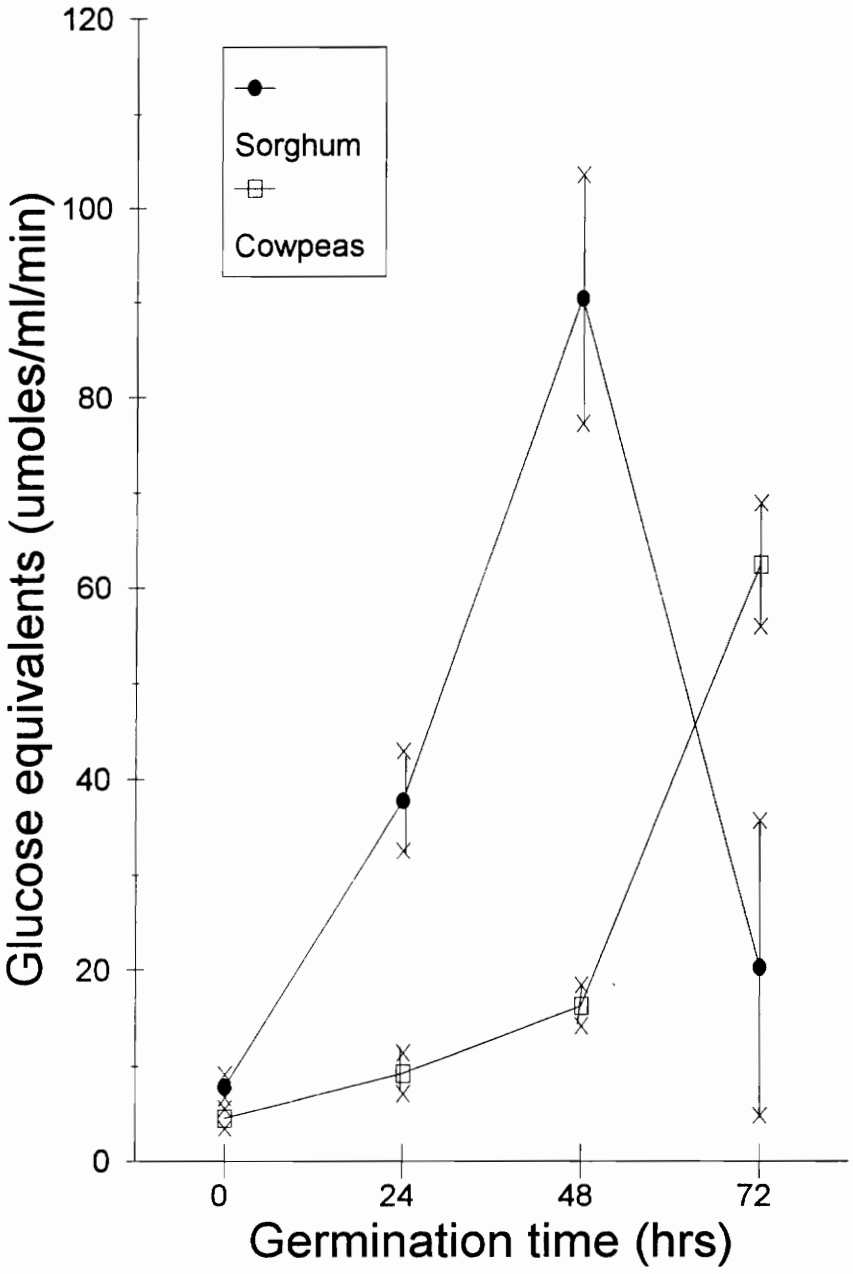


Fig. 3. Germination effect on glucanase activity

cell wall degradation may be limited by the large quantities of protein (60%) associated with isolated cell walls of sorghum (Etokakpan and Palmer, 1990).

Etokakpan and Palmer (1990) incubated 2.5 ml of crude sorghum enzyme extracts with 11 mg of dried sorghum cell walls for 8 hours at 37°C. Solubilized carbohydrates were analyzed using high pressure liquid chromatography (HPLC). Results indicated that sorghum has low endo-(1,3)(1,4)-ß-D-glucanase activity due to its low hydrolysis of the cell walls. Similarly EtokAkpan (1992) reported very low endo-(1,3)(1,4)-ß-D-glucanase activity in four varieties of sorghum grown in Nigeria.

Degradation of ß-glucan occurs following release from endosperm cell walls by carboxypeptidase at the onset of germination. Dissolved ß-glucan is degraded by endo-(1,3)-glucanase to give a product that can be attacked by endo-(1,3)(1,4)-ß-D-glucanase and endo-ß-(1,4)-glucanases. The products formed are cellobiose, laminaribiose and 3-O-D-cellobiosyl-D-glucose. Finally these products will be degraded to glucose by ß-glucosidases, cellobiase and laminaribiase (Etokakpan, 1992). Low endo-(1,3)(1,4)-ß-D-glucanase activity in sorghum has been attributed to low ß-glucan content, while in cowpeas the endosperm is removed with the testa during soaking (Kadam et al, 1989). The slight increase in endo-(1,3)(1,4)-ß-D-glucanase activity in cowpeas (Figure 3) during germination could due to the growing acrospires. ß-glucan is found in leaves, stems, husks, coloeptiles and roots of cereals and other monocotyledons (Anderson et al, 1978).

**c. Drying effect and enzyme activity**

**1. *α*-Amylase activity**

In both malted sorghum and cowpea flours, drying significantly affected *α*-amylase activity ($P<0.05$) (Tables 9 and 10). No significant difference ($P<0.05$) was obtained between 5 to 10 hrs of drying (Figures 4) on sorghum and cowpea *α*-amylase activity. The only significant difference

Table 9. Drying effect on sorghum enzyme activity[x] (*μ*moles reducing sugar/ml/min)

| Drying time at 60°C (hrs) | *α*-amylase | Glucanase |
|---|---|---|
| 2.5 | 494.24[b] ± 65.26 | 17.67[c] ± 8.97 |
| 5 | 599.98[a] ± 79.83 | 60.51[a] ± 8.66 |
| 7.5 | 610.12[a] ± 86.25 | 48.39[ab] ± 15.93 |
| 10 | 618.81[a] ± 81.11 | 29.59[bc] ± 7.01 |
| P-value | 0.0001 | 0.0017 |
| F-value | 7.96[a] | 5.27 |

[x]Dry weight values and means of 48 observations ± SE

[a,b,c,d] Means in the same column with different letters are significantly different at $P < 0.05$.

Table 10. Effect of drying on cowpea enzyme activity[x] (*u*moles reducing sugars/ml/min)

| Drying time at 60°C (hrs) | *α*-amylase | Glucanase |
|---|---|---|
| 2.5 | 297.58[b] ± 25.31 | 21.24[ab] ± 6.23 |
| 5 | 414.75[a] ± 42.04 | 34.66[a] ± 7.50 |
| 7.5 | 383.89[a] ± 41.81 | 17.04[b] ± 2.78 |
| 10 | 376.74[a] ± 44.15 | 19.65[b] ± 3.45 |
| P-value | 0.0004 | 0.0665 |
| F -value | 6.47 | 2.43 |

[x] Dry weight values and means of 48 observations ± SE

[a,b,c] Means followed by different letters in the same column are significantly different at $P < 0.05$.

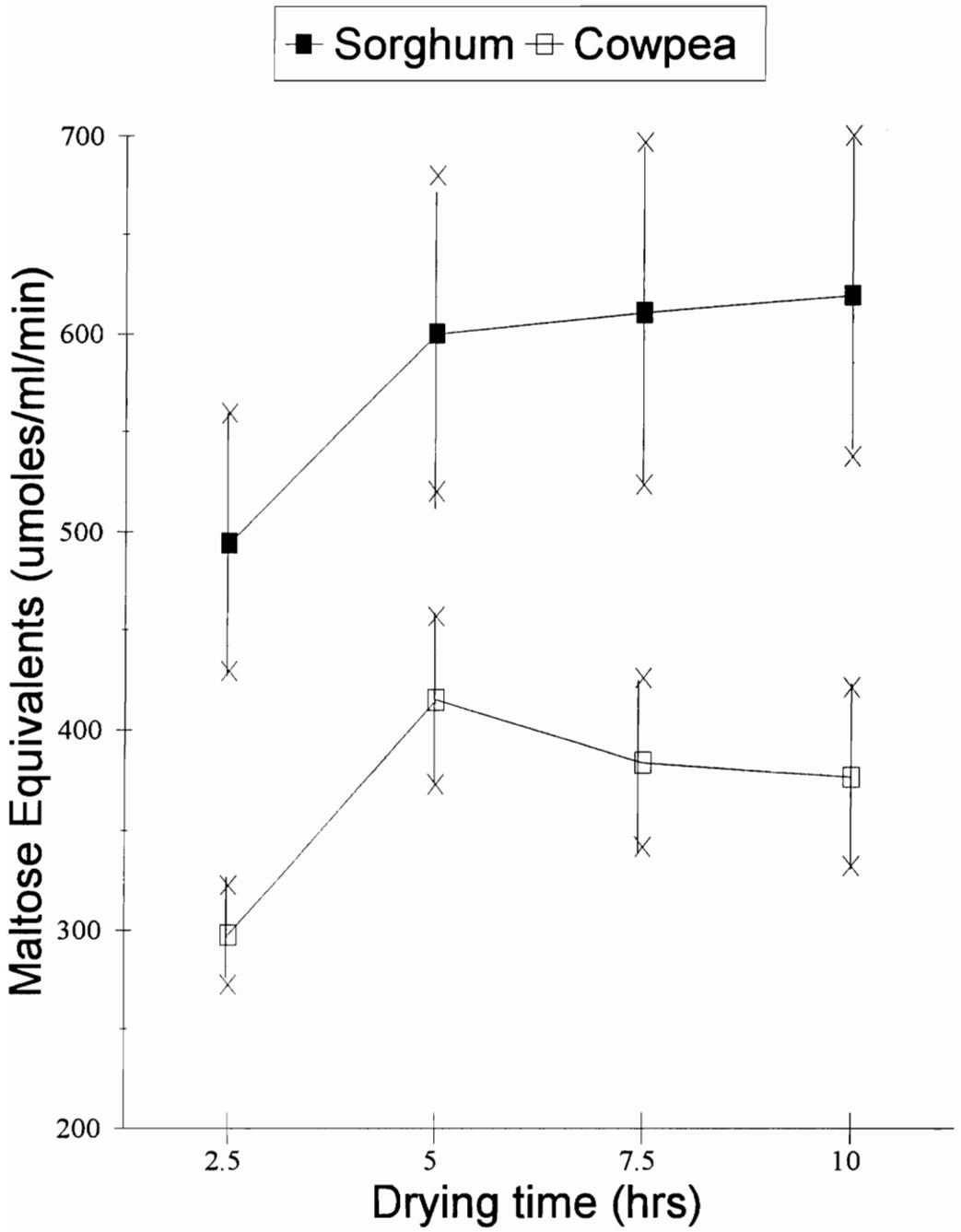


Fig. 4. Drying effect on alpha-amylase activity

occurred at 2.5 hrs, where lower $\alpha$-amylase activity was observed (Figure 4). These results differ from those of Morall et al (1986) who found loss of diastatic power in germinated sorghum samples containing high moisture. High moisture was defined as samples that were germinated in sufficient water so that none drained off the malt and the grain felt wet. The difference in results could be attributed to variations in experimental methods. For example, Morall and his co-workers (1986) steeped their samples for 16 hrs and dried for 24 hrs. They also included the dried rootlets in their analysies. All these factors will influence enzyme activity.

After 2.5 hrs drying, the average moisture content was 29.81% in cowpeas and 11.44% in sorghum (Table 11 and 12). At 10 hrs the moisture content had dropped to 4.62% and 6.74% respectively (Table 11 and 12). The combined action of germination and drying had a significant effect on sorghum $\alpha$-amylase activity (Figure 5). Similar results were obtained for cowpea $\alpha$-amylase activity (Figure 6). These results indicate that enzyme activity was retained during prolonged dehydration. Owuama and Asheno (1994) reported malts with over 10% moisture when subjected to elevated temperature treatments, accelerated inactivation of enzymes takes place. Greater enzyme survival can occur at higher temperatures if the malt is less moist.

Retention of enzyme activity could be attributed to the low kilning temperature (60°C) used or to continued germination during drying. For example, Bathgate (1973) reported malt dried at 35-55°C for over 24 hrs to a moisture content of 8% still retained 75% of its germinative power. He also reported that when moisture content was less than 30%, some enzymic activity continued as long as the temperature did not exceed 60°C (Bathgate, 1973).

Pathirana et al (1983) dried sorghum "green malt" at 45°C to 10% moisture level and further kilned the malt at temperatures of 80, 100, 120 and

Table 11. Percent moisture content in cowpeas

| Drying time (hrs) | G = 0 | G = 24 | G = 48 | G = 72 | MEAN G |
|---|---|---|---|---|---|
| 2.5 | 27.64 | 30.31 | 20.33 | 40.33 | 29.81 |
| 5 | 7.29 | 6.96 | 8.87 | 17.65 | 10.19 |
| 7.5 | 5.93 | 4.38 | 5.83 | 7.58 | 5.93 |
| 10 | 4.9 | 3.75 | 4.21 | 5.62 | 4.62 |
| Mean | 11.44 | 11.50 | 9.81 | 17.80 | |
| P - Value | 2.69E-05 | | | | |
| F - Value | 35.11 | | | | |

Table 12. Percent moisture content in sorghum

| Drying time (hrs) | G = 0 | G = 24 | G = 48 | G = 72 | Mean G |
|---|---|---|---|---|---|
| 2.5 | 9.95 | 9.70 | 11.70 | 14.41 | 11.44 |
| 5 | 7.88 | 6.63 | 8.19 | 8.67 | 7.84 |
| 7.5 | 7.50 | 7.16 | 7.44 | 6.87 | 7.24 |
| 10 | 6.76 | 6.65 | 6.7 | 6.84 | 6.73 |
| Mean | 8.02 | 7.53 | 8.50 | 9.19 | |
| P - Value | 0.000693 | | | | |
| F - Value | 15.36 | | | | |

P = 0.000693 and F = 15.36 for row values.

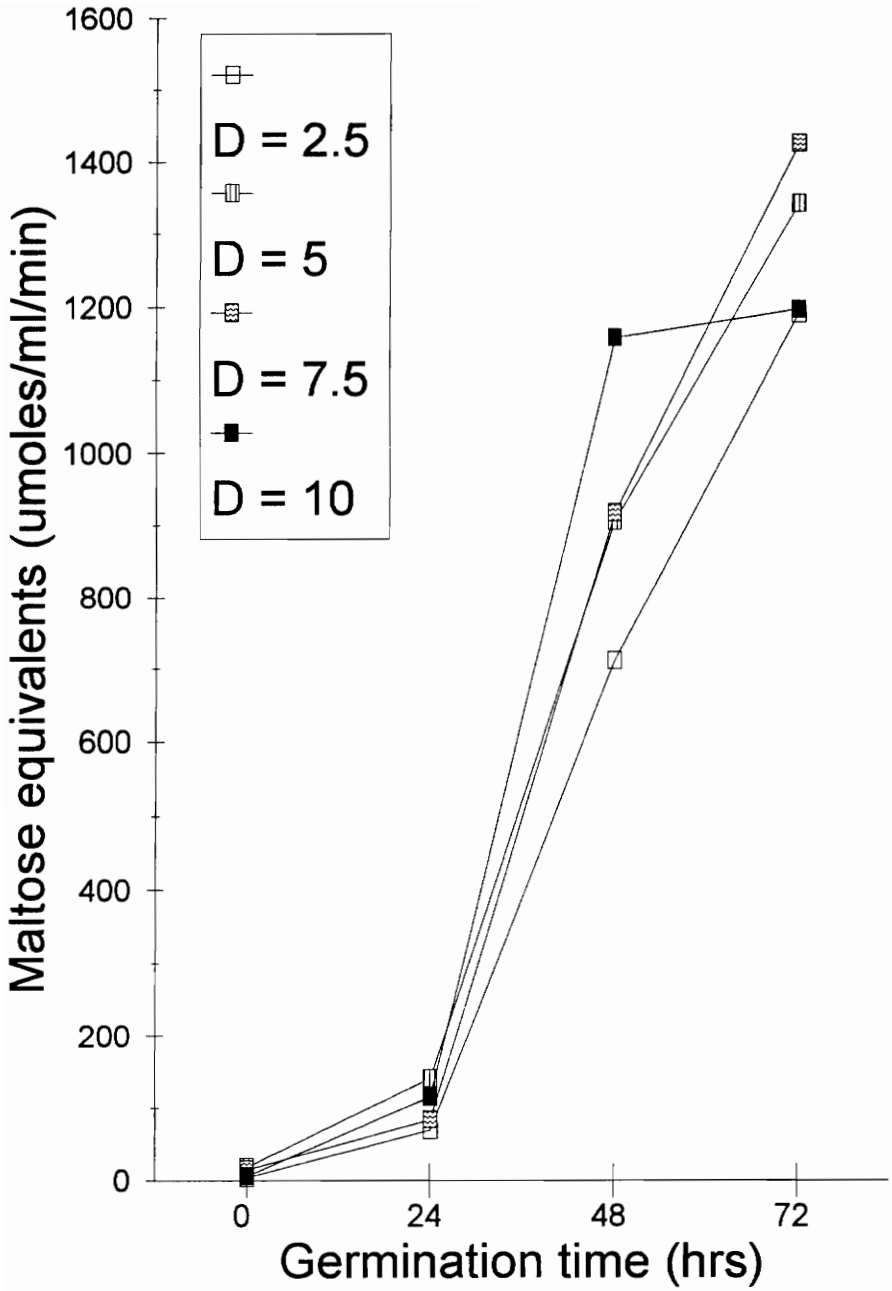


Fig. 5. Effect of germination and drying on sorghum alpha-amylase activity

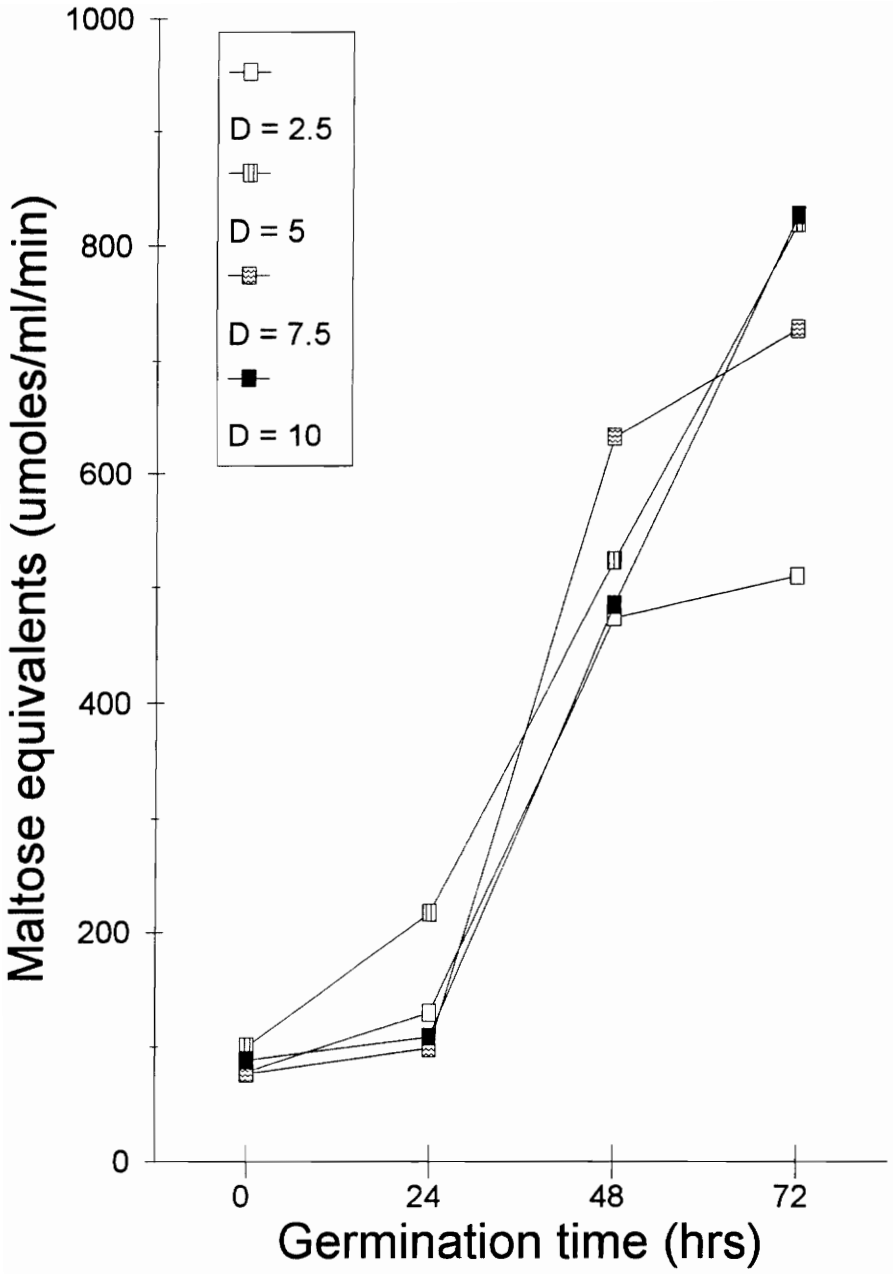




Fig. 6. Effect of germination and drying on cowpea alpha-amylase activity

140°C for periods of 2, 3, 4, 5 and 6 hrs. They reported loss of α-amylase activity at 120 and 140°C. However, a slight decrease in liquefying power occurred between 80 and 100°C, showing α-amylase was much more resistant to heat. Gopaldas et al, (1988) studied the effect of different drying methods (sun-drying at 40°C, oven drying at 50°C and toasting at 80°C), on wheat α-amylase activity. They found activity from sun-dried and oven dried samples were comparable. A drastic decrease in activity occurred in samples toasted at 80°C.

In the brewing industry, increases in α-amylase activity during kilning is encouraged and results in the formation of "crystal malt". In crystal malt, α-amylase activity increases while still on the kiln and reduces the entire endosperm to a sugary mass. Subsequent heating at higher temperatures (roasting at 70 and 80°C), results in the formation of color and a high flavor profile. The increase in malt sugar content is needed as a source of fermentable sugar during alcohol production. Malt produced from low kilning temperatures retain their enzyme activity and are used in infant foods as a source of flavor, sweetness, nutrition and enzymes (Bevan, 1988). Thus the manipulation of malting conditions will produce different types of malt which can be used in a wide range of food products.

**2. Endo-(1,3)(1,4)-ß-D-glucanase**

Drying had no significant effect ($P > 0.05$) on endo-(1,3)(1,4)-ß-D-glucanase activity in cowpeas (Figure 7 and Table 13). However, drying significantly affected sorghum endo-(1,3)(1,4)-ß-D-glucanase ($P < 0.05$) (Figure 7). In sorghum, only 5 and 7.5 hrs were not significantly different in value (Table 6). The combined effect of germination and drying in sorghum endo-(1,3)(1,4)-ß-D-glucanase activity was significant (Table 14).

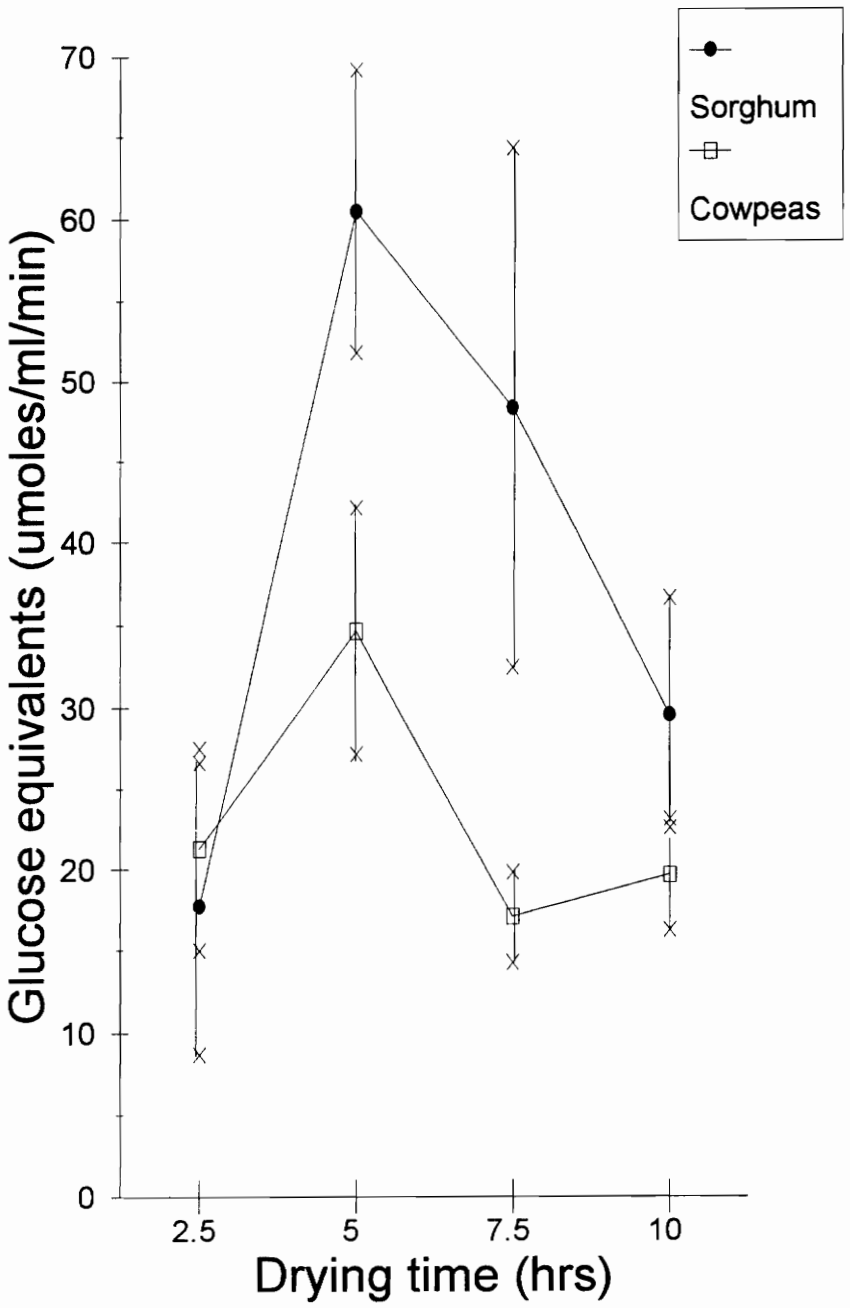


Fig. 7. Drying effect on glucanase activity

Table 13. Effect of drying and germination on cowpea glucanase activity[x] (μmoles glucose/ml/min)

| Drying time (hrs) | G = 0 | G = 24 | G = 48 | G = 72 |
|---|---|---|---|---|
| 2.5 | 12.01 ± 3.21 | 11.40 ± 0.74 | 15.85 ± 1.37 | 89.17 ± 18.75 |
| 5 | 3.848 ± 3.17 | 15.24 ± 3.99 | 26.46 ± 4.10 | 120.03 ± 12.25 |
| 7.5 | 1.50 ± 0.79 | 11.91 ± 2.94 | 21.27 ± 3.01 | 46.65 ± 4.98 |
| 10 | 6.645 ± 1.04 | 10.84 ± 2.23 | 23.00 ± 5.42 | 47.07 ± 8.21 |
| P-value | 0.0732 (NS) | | | |
| F-value | 1.79 | | | |

*Dry weight values and means of 24 observations ± SE

**NS** = Not significant at $\alpha = 0.05$

Table 14. Effect of drying and germination on sorghum glucanase activity[x] (*u*moles glucose/ml/min)

| Drying time (hrs) | G = 0 | G = 24 | G = 48 | G = 72 |
|---|---|---|---|---|
| 2.5 | 8.31 ± 1.45 | 13.42 ± 2.12 | 119.54 ± 21.16 | 35.68 ± 23.09 |
| 5 | 16.04 ± 1.38 | 73.85 ± 11.42 | 107.48 ± 15.18 | 92.64 ± 29.64 |
| 7.5 | 13.20 ± 3.11 | 26.14 ± 5.94 | 154.59 ± 34.44 | 43.92 ± 10.75 |
| 10 | 1.49 ± 1.06 | 45.66 ± 7.85 | 52.54 ± 9.73 | 89.76 ± 40.47 |
| P-value | 0.0001 | | | |
| F-value | 4.11 | | | |

[x] Dry weight values and means of 24 observations ± SE

# CHAPTER 5

## SUMMARY AND CONCLUSION

The influence of both germination and drying time was significant ($P < 0.05$) for all enzymes except for cowpea endo-(1,3)(1,4)-ß-D-glucanase activity. Drying time and germination time main effects were significant ($P < 0.05$) for sorghum $\alpha$-amylase, sorghum endo-(1,3)(1,4)-ß-D-glucanase and cowpea $\alpha$-amylase. Cowpea endo-(1,3)(1,4)-ß-D-glucanase showed only a significant germination effect ($P < 0.01$). Based on results obtained from Duncan's Multiple Range Test, a drying period of 5 hrs and germination period of 48 hrs would be most effective in producing higher levels of $\alpha$-amylase and endo-(1,3)(1,4)-ß-D-glucanase activity in malted sorghum and cowpea flour.

The results indicate $\alpha$-amylase is stable during the drying times of 5 to 10 hrs. Thus shorter drying times could be used in making malt, for weaning food production. In the context of Third World countries, shorter drying times will translate to lower utilization of fuel. As the brewing industry in these nations is more developed, the possibility of malt production geared towards formulation of nutritional products should be further investigated and encouraged.

The results obtained in this study reveal the importance of determining conditions for optimum hydrolase activity in starch based food systems for developing countries. Optimum conditions will provide malts that can be used to minimize factors common to weaning foods in developing countries, such as; high viscosity, low caloric density and poor sensory characteristics.

# CHAPTER 6

# FURTHER STUDIES

The statistical analysis obtained from experimental data indicated how significantly enzyme activity was affected by germination and drying. It is felt that it would be beneficial to purify and characterize the enzymes from germinated samples, especially in legumes due to the existence of ample research on cereal systems. Other possibilities for future research are:

* To vary malting parameters such as steeping time and drying temperature on enzyme activity
* To study the effect of kilned versus unkilned (freeze-dried) samples on enzyme activity
* To determine the optimum enzyme extraction time for determining enzyme activity
* To study the effect of germination and drying on protein and starch content
* To determine protein efficiency ratio (PER) at various cereal to legume ratios
* To perform microbial tests for determination of storage stability and safety of germinated flour
* To study how water activity ($A_w$) is influences enzyme activity and
* To compare different cultivars of legumes or cereals.

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