A study of glucose storage polymers: teosinte starch, starch crystallinity, and cyanobacterial glycogen

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A Study of Glucose Storage Polymers: Teosinte Starch, Starch Crystallinity, and Cyanobacterial Glycogen

by

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A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Food Science and Technology

Program of Study Committee:
Jay-lin Jane, Major Professor
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Iowa State University

Ames, Iowa

2001

Graduate College
Iowa State University

This is to certify that the Master's thesis of

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has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy

DEDICATION

I dedicate this thesis to my beloved husband, Nicholas, for your friendship, love, and encouragement. You are everything that I could have hoped for—my confidant, kitchen buddy, running partner, and best friend. You have done so much for me, and this is only the beginning.

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ABSTRACT

This collection of studies focused on characterization of maize and teosinte starches, creation of a model system for studying starch crystallinity, and examination of cyanobacterial glycogen accumulation in various media conditions.

Chalco teosinte, BSSS maize, BSSS maize-Chalco teosinte cross, and commercial normal maize starches all exhibited A-type X-ray diffraction patterns, similar degrees of crystallinity, amylopectin molecular weights, and amylose contents. Chalco teosinte starch granules were small, broken, or hollow; other starch granules were spherical or polygonal. Average branch chain length was smallest for Chalco teosinte starch (degree of polymerization (dp) 23.5) and largest for commercial normal maize starch (dp 25.3). Differential scanning calorimetry analysis showed that Chalco teosinte starch had the lowest onset gelatinization temperature (61°C) and enthalpy change of gelatinization (11.4 J/g), but the highest rate of retrogradation (56.2% in 7 days). Pasting properties determined using a Rapid Visco Analyzer differed widely. Chalco teosinte starch had the lowest peak viscosity (135.8 RVU), and BSSS maize-Chalco teosinte cross starch had the highest (187.3 RVU).

Starch is cold-water insoluble and semi-crystalline, but glycogen is considered cold-water soluble and non-crystalline. Starch crystallinity was studied with a starch model system prepared from amorphous waxy-maize starch and highly crystalline normal-maize starch Naegeli dextrin (83% crystalline). These components, and mixtures thereof, were analyzed by X-ray diffraction and differential scanning calorimetry (DSC). Degree of crystallinity, determined by X-ray diffraction, increased linearly with increasing amounts of Naegeli dextrin. However, enthalpy

change of melting and melting temperature, determined by DSC, demonstrated a second order relationship.

Glycogen production by cyanobacteria (*Synechocystis* PCC 6803) was studied using media conditions that varied in glucose and nitrogen contents, and glycogen was extracted with ethanol only or ethanol and trichloroacetic acid. Glycogen accumulation was the highest in glucose-abundant nitrogen-deficient media (30 mg/L), but growth rate was the highest in glucose and nitrogen-abundant media. Average branch chain length ranged from dp 9.5 (glucose and nitrogen-abundant media) to dp 11.2 (glucose-abundant nitrogen-deficient media). Thus, media nutrient content influenced cyanobacterial growth rate, amount accumulated, and molecular structure of glycogen. Minor differences were observed between glycogen samples extracted with ethanol only and with ethanol and trichloroacetic acid.

GENERAL INTRODUCTION

Starch has a unique role in nature because it is the major link between sunlight and human food. Plants use solar energy to produce glucose through the process of photosynthesis, store glucose in the form of semi-crystalline starch granules, and humans consume starch-containing plants. Starch is generally made of two different types of glucose polymers: amylose and amylopectin. However, starches may contain only amylopectin (waxy starch) or amylose, amylopectin, and an intermediate component (high-amylose maize starch). Amylose is primarily a linear molecule made of glucose monomers linked α-1-4 with a few branches linked α-1-6. Amylopectin also is made of glucose chains linked α-1-4 with α-1-6 branch linkages, but it is much more highly branched and has shorter chains than amylose. Starch intermediate component displays characteristics between those of amylose and amylopectin. The semi-crystalline structure of starch distinguishes it from glycogen, a glucose storage molecule in mammals, bacteria, fish and other living organisms. Glycogen is water-soluble and is even more highly branched than amylopectin.

People all over the world have carefully cultivated starch-producing plants that grow well in the region where they live, and the crop of choice in North and South America is maize or corn. Maize was grown only in the Americas before 1492 when Columbus and his men discovered it in Cuba. Since the discovery of maize, many geneticists, agronomists, and botanists have attempted to determine the origin of maize. Teosinte, the closest relative of maize, has constantly been in the discussion. There are several hypotheses about the relationship between maize and teosinte. Dr. George Beadle upheld the hypothesis that teosinte

gradually evolved into maize, and this is currently the most widely accepted theory of the origin of maize.

All teosinte research was originally done in Mexico because light sensitivity limited the growth of teosinte to regions with less than 12 hours of daylight. However, Dr. Beadle overcame this obstacle by breeding a daylight-adopted Chalco teosinte variety that grows in the Midwest United States. Dr. Beadle presented the results of his work at the 1977 annual American Seed Trade Association meeting where he also gave away samples of his corn belt-adopted teosinte. Dr. Howard Smith received a sample at this meeting, returned to the Iowa State University Agronomy Department, and made a cross between the Chalco teosinte and BSSS maize (a variable breeding line). The cross was randomly mated for several years by Dr. Smith and later by Dr. Pollak.

Much research has been done to establish the genetic similarities and differences between maize and teosinte, but there are no reported studies examining the physicochemical properties of teosinte starch. Therefore, Chalco teosinte starch was characterized and compared with maize starches, including commercial normal maize, BSSS maize, and the maize-teosinte cross provided by Dr. Linda Pollak (described above). Chalco teosinte was chosen because it is the most maize-like variety of teosinte. The goals of this study were to characterize Chalco teosinte starch and compare it with normal-maize starch and to determine if crossing maize and teosinte resulted in maize starch with unique or desirable properties.

Starch granules display semi-crystalline properties, meaning that they contain both crystalline and amorphous regions. X-ray diffraction is commonly used to characterize the type of crystal pattern in starch granules (A, B, or C-type) and to quantify the amount of crystalline material in starch granules. Starch thermal properties (gelatinization, or melting temperature and

enthalpy change of gelatinization) are often determined by differential scanning calorimietry (DSC). Starch studies rarely include a comparison between the degree of crystallinity determined by X-ray diffraction and DSC data. However, synthetic polymer studies often use enthalpy change of melting (determined by DSC) as a measure of crystallinity. The goal of this study was to create a starch model system using amorphous waxy maize starch and normal maize starch Naegeli dextrin (acid hydrolyzed starch, consisting of 83% crystallite) to study the correlation between degree of crystallinity, enthalpy change of melting, and melting temperature.

Glycogen is a glucose polymer used for energy storage in many living organisms, including cyanobacteria, or blue-green algae. The amount and structure of glycogen stored in a bacterium depend on the media growth conditions and glycogen extraction procedures can influence the amount and structure of purified glycogen. For example, media rich in glucose but deficient in nitrogen, sulfate, or phosphate have been shown to increase bacterial glycogen accumulation. This study was performed to determine how the molecular structure and amount of glycogen in cyanobacteria (*Synechocystis* PCC 6803) were affected by glucose and nitrogen contents in the growth media, and to determine what effect trichloroacetic acid (used for protein precipitation during extraction) had on glycogen structure.

Thesis Organization

This thesis contains a general introduction and literature review followed by three chapters describing three separate experimental studies. The first chapter, "Characterization of Chalco Teosinte and Maize Starches" will be submitted to Cereal Chemistry Journal for publication. The second chapter, "Crystallinity and Melting Properties of a Starch Model

System" describes work that was initially done to establish a standard operating procedure for starch crystallinity calculation, but later became an experimental investigation on its own. Further refinement of these experiments would be required for publication. The third chapter, "Effects of Growth Conditions on Amount and Structure of Glycogen Produced by Cyanobacteria, *Synechocystis* PCC 6803" describes work done in collaboration with Sang-ho Yoo. This study was done to optimize growth conditions for bacteria used in Dr. Yoo's dissertation research, and therefore it is not considered publishable as-is. General conclusions complete this thesis.

LITERATURE REVIEW

Starch

General Properties

Glucose, a product of photosynthesis, is stored as starch polymers in granules in plant stems, roots, leaves, tubers, seeds, and fruits (Robyt 1998). The starch granule is an intricately organized yet widely variable energy storage system. Anhydroglucose monomers are arranged in chains linked by α-1-4 bonds with branches attached by α-1-6 bonds. Variation in the length of the linear chains and frequency of branching results in the two main types of molecules in starch granules: amylose and amylopectin. Amylose has long linear chains and few branches while amylopectin has relatively short linear chains and many branches, and a much larger molecular weight. A third component, intermediate between amylose and amylopectin, has been found in some starches (Lansky 1949, Peat et al 1952, Kasemsuwan et al 1995). Some starch granules also contain small amounts of lipid and phosphorous. Starch is the major carbohydrate source in the human diet and is usually consumed in the form of cereal products such as rice, potatoes or wheat bread. However, starch displays great versatility as a food ingredient and can be found in a wide variety of food products from chocolate milk to ground meat (Charley and Weaver 1998).

Amylopectin

Amylopectin is generally considered to be the major component of starch, but starches can vary widely in their amylopectin contents. For example, normal maize starch contains 70% amylopectin while waxy maize and high-amylose maize starches contain 100, and 20-50% amylopectin, respectively (Johnson 2000). Amylopectin molecules contain glucose units bonded by α-1-4 linkages with approximately 5% α-1-6 branch points, and an average branch chain

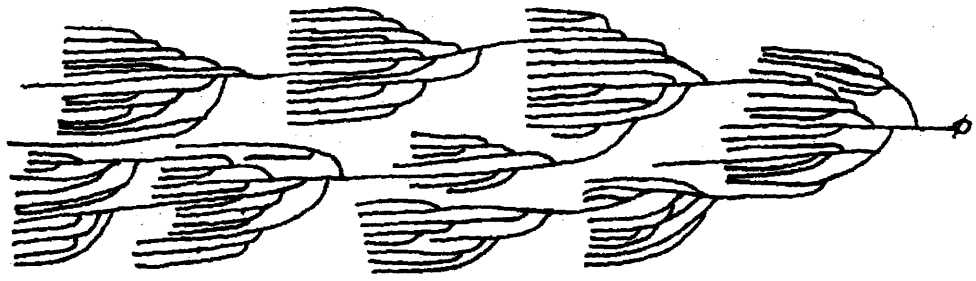
length of degree of polymerization (dp) 20 (Whistler and Daniel 1984, Robyt 1998). The molecular weight of amylopectin ranges from 10^7 to 10^8, which is much larger than the molecular weight of amylose (approximately 10^6) (Whistler and BeMiller 1997).

Amylopectin molecules are made of A, B, and C chains. A chains are attached via the reducing end in α-1-6 branch linkages to B chains or the C chain. B chains are attached to other B chains or the C chain, just as A chains are, but they differ in that they carry A or B chains. There is only one C chain per amylopectin molecule, and this chain is distinguished from the others because it carries a reducing end. The A to B chain ratio is referred to as the degree of multiple branching (Manners 1985), and this ratio has been used to study amylopectin structure (Altwell et al 1980). Many different models of amylopectin molecular structure have been proposed, but the most widely accepted is the cluster model by French (1972), Figure 1A. Hizukuri (1986) defined this model further, as shown in Figure 1B, and this is the currently accepted model of amylopectin structure.

Amylose

Amylose is the minor component in most starches, accounting for 30% of normal maize starch, but it makes up 50 to 80% of high-amylose maize starches (Johnson 2000). Amylose differs from amylopectin in branching pattern, chain length, and molecular weight. Until Peat et al (1949) discovered that β-amylase hydrolysis of amylose was incomplete (70%) amylose was thought to be completely linear. Complete hydrolysis was accomplished with the addition of pullulanase, indicating the presence of some α-1-6 branch linkages (Hizukuri et al 1981, Takeda and Hizukuri 1989). Further studies showed that amylose contains an average of 2.9 branch chains per molecule; the average normal maize amylose molecule is made of 990 glucose units (Takeda et al 1988, Suzuki et al 1994).

A



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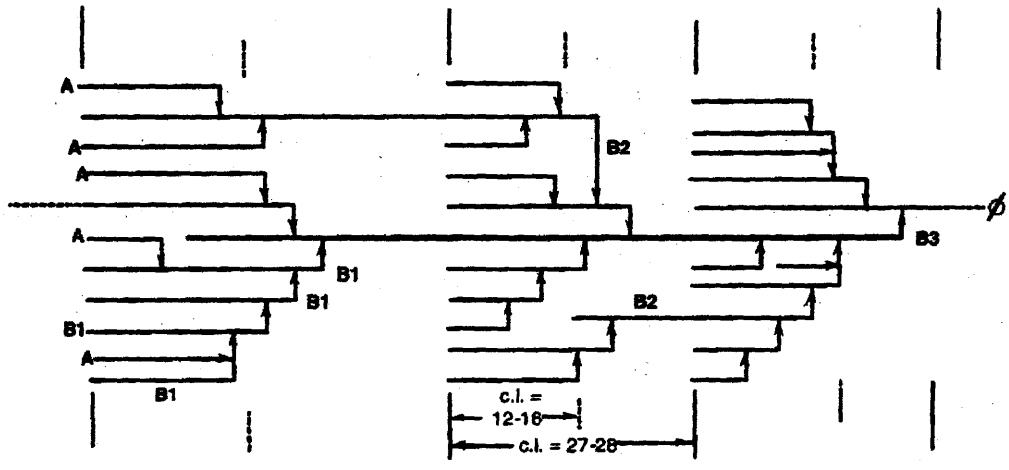

Figure 1. Molecular models for amylopectin structure. The cluster model proposed by French (1972) (A) and cluster model with further definition by Hizukuri (1986) (B).

Amylose exists as random coils in neutral, aqueous solutions, but easily forms helical complexes of 6 to 8 glucose units per turn with complexing agents such as alcohols or fatty acids (Rundle and French 1943, Banks et al 1971, Davies et al 1980, Jane and Robyt 1984). The tendency of amylose to complex with n-butyl alcohol allows for separation of amylose and amylopectin, and blue amylose-iodine complexes allow amylose to be quantified spectrophotometrically (Schoch 1964, Banks et al 1974). Complexed amylose exhibits an X-ray diffraction pattern known as a V-pattern (Winter and Sarko 1974, Davies et al 1980).

Intermediate Component

Lanskey et al (1949) reported the discovery of a starch component with iodine-binding capacity and β-amylolysis limits between those observed for amylose and amylopectin. The amount and fine structure of this component varies with the maturity and genetic background of its source (Banks et al 1974, Wang et al 1993, Kasemsuwan et al 1995, Hizukuri 1996). As the degree of branching and molecular weight definitions of amylose and amylopectin are not completely clear, it is difficult to exactly define intermediate components. Further studies are needed in this area.

Granules

Amylose, amylopectin, and intermediate components of starch are packed into semi-crystalline structures called granules (French 1972, Hood 1982). These structures are complex arrangements of macromolecules, yet they are relatively tiny. One pound of maize starch contains approximately 800 billion granules (Charley and Weaver 1998). Figure 2 shows a model of how amylose and amylopectin might be arranged in a starch granule, as proposed by Lineback (1984). The size and shape of starch granules vary greatly. Starch granules range in size from 0.5 to 100 μm in diameter, and can be round, oval, or polygonal with smooth or rough surfaces,

as revealed by scanning electron microscopy (Jane et al 1994). Light microscopy is also a useful tool for studying starch granular structure. Under polarized light, starch granules demonstrate birefringence patterns known as Maltese crosses. The center of the Maltese cross corresponds to the granule hilum, which is thought to be the starting point of biosynthesis (French 1984). Jane and Shen (1993) found that amylose molecules are present through the granule, but are more concentrated at the periphery than the core of the granule. Growth rings are concentric circles of alternating more and less densely packed material that have been found in starch granules by examination under a light microscope (Eliasson and Gudmundsson 1996).

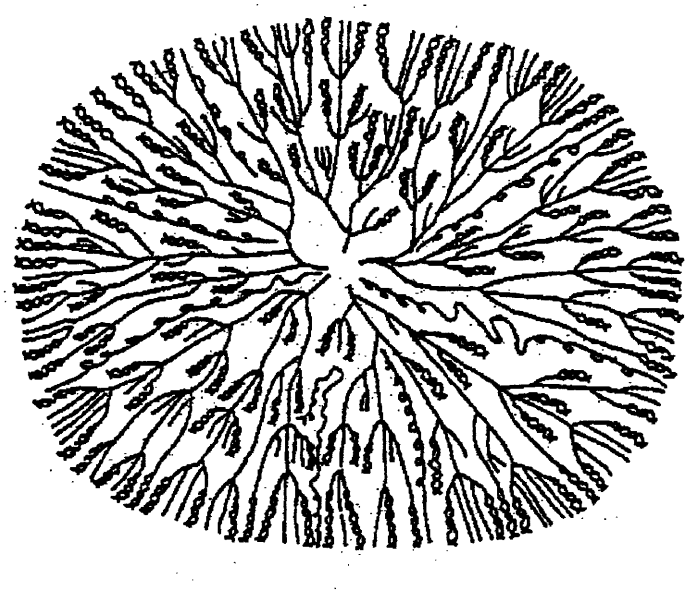

Figure 2. Model of starch granule proposed by Lineback (1984).

Starch granules can be classified by their X-ray diffraction patterns into A, B, and C-type starches. A-type starches have shorter average branch chains than B-type starches (Hizukuri et al 1983, Hizukuri 1985, Jane et al 1999). Jane et al (1999) found that A-type starches generally

have shorter peak chain lengths and larger proportions of short chains than do B-type starches. C- type starches are intermediate between A and B-type starches (Wu and Sarko 1977). Normal-maize starch is an example of an A-type starch; potato starch is an example of a B-type starch. The unit cell structures of A and B-type starches are shown in Figure 3.

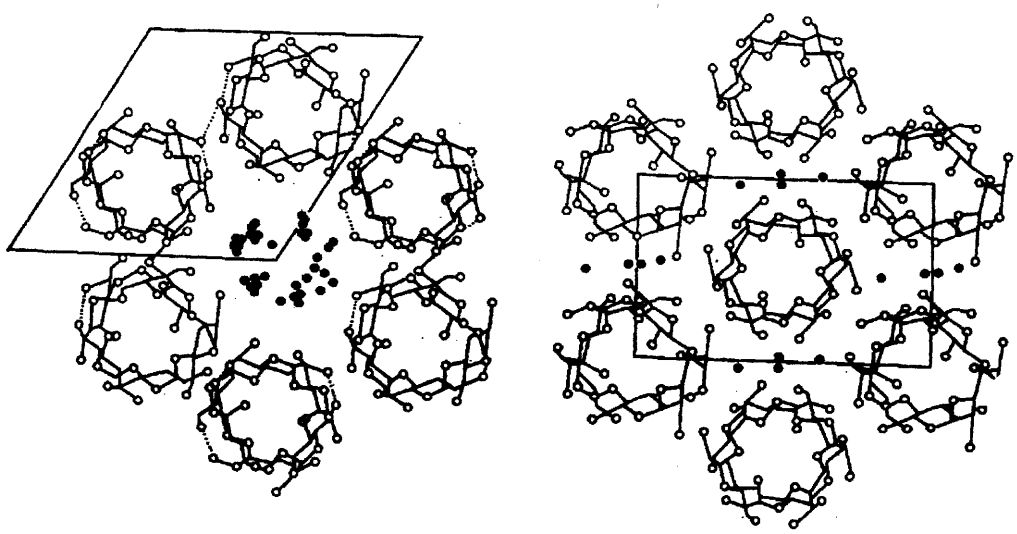

Figure 3. Unit cell and helix packing of B-type (left) and A-type (right) starches (Wu and Sarko 1978 a, b).

Minor Components

Phosphorous and lipids are the most common non-starch minor components found in starch granules. These minor components can significantly affect the physical properties of starches, even though their concentrations are small. Starch granules contain surface lipids, or non-starch lipids, and internal lipids, also known as monoacyl lipids (Morrison 1988). Cereal starches contain internal lipids in the form of free fatty acids and lysophospholipids (Schoch 1942, Morrison 1988), but there are not significant amounts of lipids in root or tuber starches

(Swinkels 1985, Morrison 1988). Some evidence suggests that internal lipids are present in the amorphous region of the granule (Morrison et al 1993). Phosphate monoesters and phospholipids are the two main forms of phosphorous found in native starch granules. Phosphate monoesters are the most prominent form of phosphorous in root and tuber starches whereas phospholipids are the most prominent form in cereal starches (Hizukuri et al 1970, Tabata et al 1975). Waxy cereal starches are an exception because they generally contain substantially less phosphorous than normal starches, and the phosphorous present is in the form of phosphate monoesters rather than phospholipids (Lim et al 1994). These two forms of phosphorous in starches have opposite effects on the starch pasting properties. Phospholipids have been shown to decrease peak viscosity (Medcalf et al 1968) and increase paste opacity (Swinkles 1985), but phosphate monoesters increase paste clarity and peak viscosity (Hamilton and Paschall 1967).

Gelatinization and Retrogradation

Gelatinization of starch granules occurs when starch is heated in water, and is evidenced by irreversible loss of birefringence, and loss of crystallinity (French 1984). Different starches gelatinize at different temperatures. Jane et al (1992) correlated amylopectin branch chain length to gelatinization temperature, and found that longer branch chain lengths result in higher gelatinization temperatures. Many authors have proposed that gelatinization temperatures depend on granule size (Banks and Greenwood 1975), degree of crystallinity (Zobel 1984), presence of lipids (Morrison 1988) and phosphorous derivatives (Lim and Seib 1993).

After gelatinization is complete, continued heating in water results in starch pasting. Stored gelatinized starch or starch paste undergoes a process known as retrogradation. During retrogradation, gelatinized starch molecules reassociate into an ordered structure (Atwell et al

1988). Retrogradation happens promptly when linear amylose molecules align and re-associate; amylopectin also involves the retrogradation process (Schoch and French 1949, D'Appolonia and Morad 1981).

Crystalline Properties

The crystalline properties of starch were discovered by X-ray diffraction around 1920 by Scherrer, and later confirmed by Herzog and Jancke (Katz 1928). Katz discovered four different diffraction patterns of starch and called them A, B, C, and V. This nomenclature is still used today (Zobel 1988). The amount of crystalline material in starch can be quantified from X-ray diffraction patterns by several methods. The most common method is to calculate the ratio of area in the crystalline peaks to total area (Hermans and Weidinger 1961). The method reported by Wakelin et al (1959), which utilizes standards of amorphous and crystalline material to create a standard curve, has also been used. Although differential scanning calorimetry is not normally used as a method for measuring starch crystallinity, Lelievre (1975) pointed out that the enthalpy change of starch gelatinization is correlated to degree of crystallinity.

Starches generally have degrees of crystallinity in the range of 15 to 45% (Zobel and Senti 1960), but Nara et al (1978) found that crystallinity measurements change with moisture content in the starch sample. Hydrolysis of the amorphous regions in starch granules with hydrochloric or sulfuric acid results in Lintner or Naegeli dextrins, respectively (Naegeli 1874, French 1972, Robin et al 1974). The residues from such acid treatment demonstrate crystallinity of approximately 80% (Buleon et al 1987) and have been used to study starch structures (Umeki and Kainuma 1981, Jane et al 1997). Amorphous starch can be prepared by gelatinizing starch in dimethylsulfoxide, hot water, concentrated salt solutions (Jane 1993), or by severe physical disruption in a ball mill (Nara et al 1978).

Maize and Teosinte

Discovery of Maize

Before November 5, 1492, maize was unique to North and South America. On this date, two Spaniards traveling with Christopher Columbus explored the interior regions of Cuba and reported the finding of a grain called "maiz." There are no references to corn or maize in ancient Greek, Indian, or Chinese languages, indicating that maize was not present in the Old World before its introduction in 1492 (Mangelsdorf 1950). Archeological evidence suggests that maize has existed for at least 7,000 years, and there were already 200 to 300 cultivated varieties of maize when Columbus and his men discovered it (Beadle 1980). Maize domestication has continued during the past 500 years, but still the origin of maize is obscure.

Teosinte

There are three major hypotheses regarding the origin of maize. All hypotheses involve teosinte, a variable wild grass found in Mexico, Guatemala, and Honduras that roughly resembles maize (Beadle 1980). Teosinte has several thin stalks growing from one root system rather than one large stalk found in maize, but both plants have tassels (male flowers) at the stalk tip and broad, flat, pointed leaves, as shown in Figure 4 (Beadle 1980). While maize plants produce one or two large ears with 500-1200 spherical or conical-shaped seeds in thin, flexible yellow seed coats (Johnson 2000), teosinte produces spikes bearing 5-6 triangular-shaped seeds in stiff, thick, brown and gray speckled seed coats (Robson et al 1976), as shown in Figure 5. Therefore, there are some distinct similarities between maize and teosinte, but there are also differences that would have required major genetic mutations to overcome. The debate over how maize and teosinte are related stems from these differences.

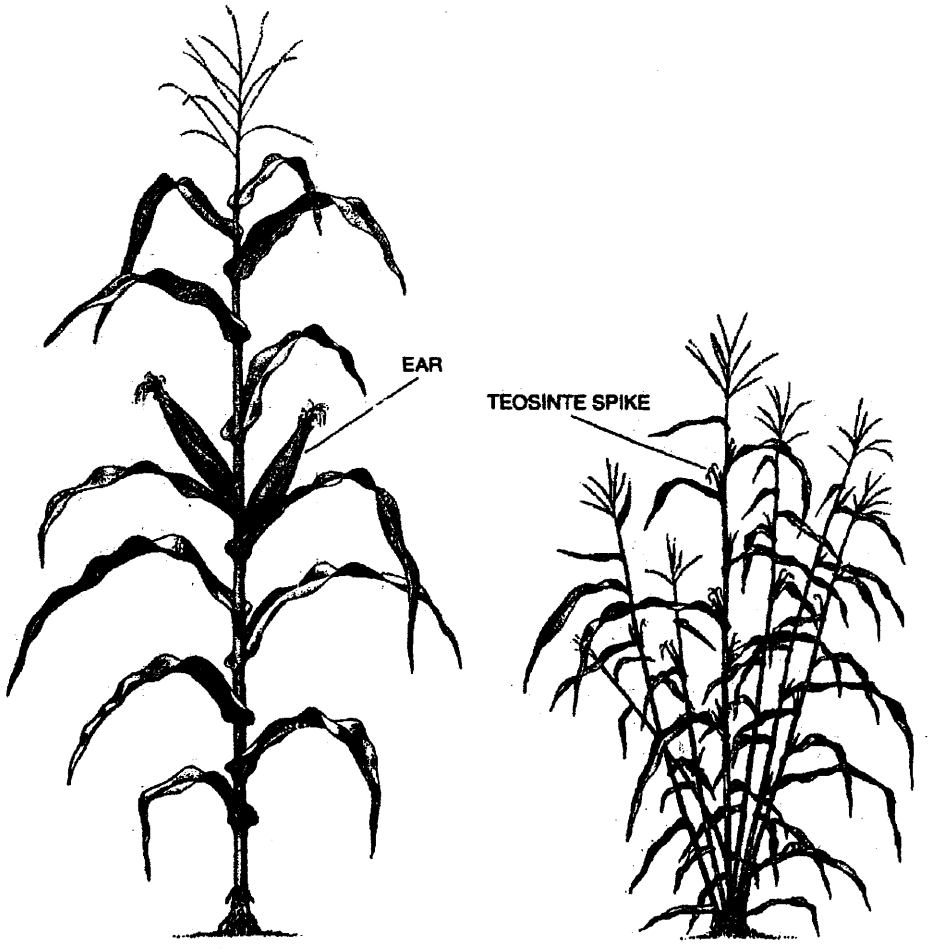

Figure 4. An artist's representation of a modern maize plant, left, and a teosinte plant, right (Beadle 1980).

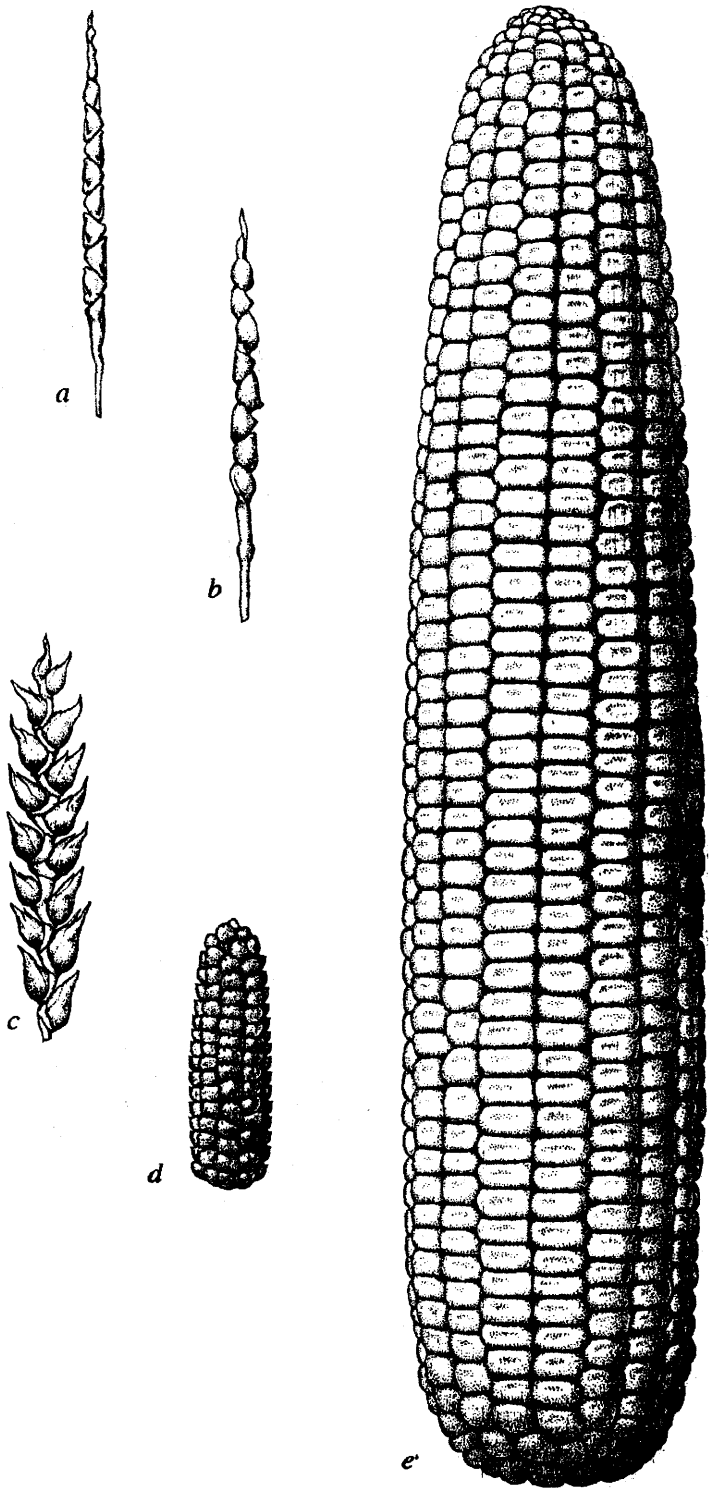

Figure 5. An artist's representation of a teosinte spike (a), a spike from a teosinte-maize cross (b), a spike of tunicate teosinte (c), an ear from a cross of teostine and modern maize (d), and an ear of modern maize (e) (Beadle 1980).

The Teosinte Hypothesis

According to the teosinte hypothesis, human selection and cultivation of teosinte resulted in maize as it is today (Beadle 1980). Folklore and the fact that both maize and teosinte have 10 chromosomes support this thesis. Ancient Aztec language refers to teosinte as *teocentli*, meaning "God's ear of corn," and teosinte is known as *madre de maiz*, or "mother of maize" in many regions of Mexico. As early as 1877, botanists believed that teosinte was the ancestor of maize according to Darwinian evolution. Mutations in spike, seed coat, and stalk structure, genes could have changed teosinte into maize. Crossing studies between maize and teosinte have provided supporting evidence for this transition (Beadle 1980). The teosinte hypothesis is currently the most widely accepted explanation for the origin of maize (Doebley 1990).

Opponents to this hypothesis site the production of teosinte-like plants from crosses between maize and tripsacum (a distant wild relative of maize) as evidence that maize did not descend from teosinte, but rather teosinte descended from maize (Mangelsdorf 1950). Iltis (1983) noted that no intermediates between maize and teosinte have been found to support the existence of a gradual evolution from teosinte to maize. Eubanks (1997) reported that progeny of crosses between perennial teositne and tripsacum demonstrated properties similar to maize, so teosinte domestication may not have been the route to modern maize.

The Pod Corn Hypothesis

The pod corn hypothesis states that modern maize descended from an extinct form of wild pod corn, and teosinte resulted from hybridization between ancient corn and tripsacum (Mangelsdorf 1950). This theory was later changed to state that teosinte resulted from hybridization between ancient (now extinct) maize and tripsacum, and modern maize resulted from hybridization between teosinte and maize (Wilkes 1979, Mangelsdorf 1983, 1986).

Recently reported success in crossing *Z. diploperennis Iltis, Doebley & Guzman* (a diploid perennial teosinte) with tripsacum to produce a plant with some maize-like qualities supported this hypothesis. According to Eubanks (1997), this crossing study indicated that tripsacum might have played an important role in the ancestry of maize. Mangelsdorf (1950) stated that the discovery of wild, primitive pod corn was the strongest evidence to support this theory.

Challengers of the pod corn hypothesis note that tripsacum and maize do not cross naturally (Beadle 1980). Man-made tripsacum-maize crosses are usually sterile, and thus could not have resulted in teosinte (Beadle 1980, Eubanks 1997). Iltis (1983) points out that Mangelsdorf (1950, 1983, 1986) did not explain the origin of the wild maize that supposedly was domesticated into modern maize. In addition, organelle DNA variation provides biochemical evidence (Timothy et al 1979) and the existence of several distinct teosinte taxa provides morphological evidence (Doebley and Iltis 1980) that teosinte could not have originated from a cross between maize and tripsacum.

The Catastrophic Sexual Transmutation Hypothesis

The catastrophic sexual transmutation hypothesis is based on the theory that species go through relatively short periods of time in which basic structural changes occur, followed by long periods of little change (Stanley 1979). Iltis (1983) suggests that modern maize resulted from a relatively short time period in which the male rather than the female inflorescence of teosinte became the female maize ear. The tips of teosinte stalks have male tassels while the inner region of the plant is hormonally female. If the lower branches were shortened by some change in growing environment, then the tassels would be in female territory rather than male, and thus would produce female rather than male flowers. This sexual change in the terminal spike upset the entire physiological balance of the plant because suddenly the female spike

became the main nutrient sink. Therefore, one large ear (found in maize) rather than several small seed spikes (found in teosinte) was formed. In order for this to occur, abnormal conditions such as cold or wet summers, short growing seasons, or cold nights must have caused shortening of the branches, and farmers must have noticed, harvested, and planted the mutated plants (Iltis 1983).

Dorweiler and Doebley (1997) reported evidence against this hypothesis. They found one gene locus (*teosinte glume architecture 1, tga1*) which controls the structure of glumes (connection between the seed and spike) in teosinte plants. When maize was modified to contain this teosinte gene, thicker, more brittle glumes resulted. The large effect of one genetic mutation was interpreted to mean that teosinte slowly evolved into maize through a series of changes involving a small number of genes, but each mutation had a large effect. Doebley and Stec (1991), Szabo and Burr (1996) supported this conclusion. The catastrophic sexual transmutation theory is based on tassel and ear glumes having the same origin, but the study by Dorweiler and Doebley (1997) showed that *tga1* changes glumes in the ear but not the tassel. Fundamental structural differences between the tassel and ear glumes that would prevent the sexual transformation are ignored by the catastrophic sexual transmutation theory (Dorweiler and Doebley 1997).

Recent Studies on Maize and Teosinte

Three theories regarding the origin of maize have been proposed and challenged, but none have been proven true or false. Recent studies comparing the genes (Doebley and Stec 1991, Doebley et al 1994, Szabo and Burr 1996, Dorweiler and Doebley 1997, Eubanks 1997, Wang et al 1999), biochemicals (Stafford 1998), and proteins (Boyer and Fisher 1984, Goldner and Boyer 1989) of maize and teosinte have increased knowledge about both plants, but have

not successfully determined the origin of maize nor the relationship between teosinte and modern maize. So, the origin of maize remains a mystery today, just as it was on the day of its discovery in 1492.

Glycogen

Physiological Function

Glycogen is the polymeric glucose molecule that acts as an energy reserve in mammals, bacteria, insects, fish, and other living organisms (Robyt 1998). The structure of glycogen is similar to that of amylopectin, the highly branched molecule of starch, in that both structures are made of α-1-4 linked glucose chains with α-1-6 linked branches. However, the degree of branching in glycogen (10%) is notably higher than that in amylopectin (5%), and glycogen is non-crystalline and mostly water-soluble while amylopectin in starch granules is semi-crystalline and cold-water insoluble. Glycogen branch chains are shorter on average (10 to 12 glucose units) than amylopectin branch chains (20 to 23 glucose units) (Marshal 1974). The high degree of branching and water-solubility of glycogen are important in its physiological function as a reserve energy source, allowing large amounts of glucose to be released quickly when needed (Voet and Voet 1995).

Molecular Structure

The precise molecular structure of glycogen has been a topic of much discussion. Haworth, Staudinger, Meyer, and Whelan all proposed structural models. The Haworth and Staudinger models were disproved by debranching studies reported by Larner et al (1952). Meyer's glycogen model showed a tree-like structure in which long chains held shorter branches, which in turn held even shorter branches, and the longest chain contained a reducing end

(Meyer and Fuld 1941). The Whelan glycogen model was a modification of the Meyer model in which all branches were randomly attached and relatively short, including the chain with a reducing glucose unit (Gunja-Smith et al 1970, Whelan 1971). This randomly branched tree model is the most widely accepted and has been supported by mathematical models (Matheson and Caldwell 1999) and enzymatic studies (Rani et al 1992).

Studies of glycogen molecular structure are complicated by the fact that glycogen structure changes depending on the growth conditions of its source. Matsiu et al (1996) studied the fine structural properties of oyster glycogen and found that the proportion of short chains (2 and 3 dp) was higher in the summer than in the winter, but the branch chain length distribution range (2-35 dp) did not change. The possibility of protein involvement with glycogen structure also complicates the study of glycogen structure. Chee and Geddes (1977) treated glycogen with disulfide bond-breaking chemicals and found that the glycogen molecular weight was affected. Also, a protein found in glycogen called glycogenin has been the center of many glycogen biosynthesis discussions (Lomako et al 1993, Alonso et al 1995, Lomako et al 1995).

Cyanobacterial Glycogen

Cyanobacteria, prokaryotes also known as blue-green algae, have been found to produce glycogen (Fogg 1956, Shively 1988). Glycogen accumulates between the thylakoids in the cell (Pankratz and Bowen 1963) during the stationary stage of growth if the growth media contains an excess amount of carbon and a limiting amount of nitrogen, sulfate, or phosphate (Preiss 1969). Weber and Wober (1975) found that the branch chain length distribution profile of glycogen from *Anacystis nidulans*, a blue-green alga, was similar to profiles of sweet corn phytoglycogen, *Escherichia coli*, *Arthrobacter sp.*, and oyster glycogens, but not amylopectin. All glycogen and phytoglycogen samples had monomodal profiles while amylopectin showed a

bimodal profile. Chao and Bowen (1971) found that the blue-green alga *Nostoc muscorum* produced glycogen in α-granules (31-65 nm) that consisted of two equal parts. Purified α-granules were 95% glucose by mass and glycogen-iodine complexes demonstrated absorption with a λ_{max} at 410 nm, similar to that observed for shellfish and rabbit liver glycogens, but not amylopectin. Fujimori et al (1995) confirmed the iodine-glycogen complex absorption results for blue-green alga glycogen.

Glycogen and Starch Research

The structural similarities between bacterial glycogen and plant amylopectin and advances in genetic research have resulted in studies using bacterial glycogen as models for starch biosynthesis. *Escherichia coli* (Guan et al 1995) and cyanobacteria *Synechocystis* PCC 6803 (Yoo et al forthcoming) were genetically altered with starch-synthesizing enzymes from plants and the glycogen structures were analyzed. Such studies have failed to produce crystalline glycogen (or amylopectin), and they have yet to fully explain the process of starch biosynthesis.

The glycogen-trimming model of starch biosynthesis (Ball et al 1996) suggests that glycogen is directly involved in the biosynthesis of starch amylopectin. According to this model, starch is synthesized by careful enzymatic trimming of randomly branched glycogen, and thus glycogen is very important in all starch-producing plants as well as the organisms that depend on it for energy reserves. However, this model does not completely explain how amylose is formed, or how amylose and amylopectin are packed together in the form of a starch granule. Much research still needs to be done in the area of starch biosynthesis to gain a complete understanding of the starch biosynthesis process.

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CHARACTERIZATION OF CHALCO TEOSINTE AND MAIZE STARCHES

A paper to be submitted to Cereal Chemistry

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Abstract

Chalco teosinte, BSSS maize, and BSSS maize-Chalco teosinte cross starches were isolated, characterized and compared with commercial normal maize starch. Maize seeds contained more starch (approximately 70%) than teosinte (approximately 25%) and protein content varied from 6.7% to 13.6% for maize and teosinte seeds. All starches exhibited A-type X-ray diffraction patterns, similar degrees of crystallinity, amylopectin molecular weights, and amylose contents. However, Chalco teosinte starch granules were small, broken, or hollow while other starch granules were spherical or polygonal. Branch chain length distributions showed that Chalco teosinte starch contained the highest proportion of short chains (18.5%) and shorter average chain length (23.5 degree of polymerization) compared with the other starches. Differential scanning calorimetry analysis showed that Chalco teosinte starch had the lowest onset gelatinization temperature (61°C) and enthalpy change of gelatinization (11.4 J/g), but the largest rate of retrogradation (56.2% in 7 days). Pasting properties, determined by using a Rapid Visco Analyzer, differed widely with Chalco teositne starch having the lowest peak viscosity (135.8 RVU), and BSSS maize-Chalco teosinte cross starch having the highest (187.3 RVU). Thus, Chalco teosinte starch differed from maize starches in some aspects, but not all.

Introduction

Zea mays (corn or maize) is a member of the grass tribe *Maydeae* and has been a very important agricultural crop in the North and South America for thousands of years (Robson et al 1976). Today, maize is grown on every continent except for Antarctica; global production exceeds 500 million tons annually (Johnson 2000). With the production of such large volumes of maize and its importance in the global economy, it is surprising that the origin of maize is still debated. One hypothesis states that human selection and cultivation of teosinte, a wild grass and the closest living relative to maize found in Mexico, Guatemala, and Honduras, resulted in maize as it is today. Archeological evidence suggests that teosinte seeds were part of ancient Mexican peoples diets. The use of teosinte in the diet may have provided motivation for people to develop it into a more productive plant, one that is now known as maize (Beadle 1980).

Teosinte seeds are encapsulated in hard, dark brown fruit cases which grow in small ears or spikes containing five or six loosely arranged seeds. Strong wind or movement causes the seeds to scatter (Robson et al 1976). On the contrary, modern maize produces large ears bearing 500-1200 seeds with soft, yellow coats and no effective mechanism for seed scattering (Johnson 2000). Wild teosinte grows in the edges of maize fields in Mexico and is believed to improve maize qualities (Wilkes 1967). Reeves (1950) found that crossing teosinte with maize inbreds produced maize with greater resistance to heat and drought damage.

Recent studies have attempted to determine the genetic relationship between teosinte and maize, and explain the morphological differences between these two plants. Dorweiler and Doebley (1997) identified the gene, *teosinte glue architecture1*, which controls the thickness and stiffness of teosinte and maize seed glumes, and Szabo and Burr (1996) mapped the loci of genes controlling several morphological traits that distinguish teosinte from maize. Eubanks (1997)

employed DNA techniques to examine the link between maize and its wild relatives, teosinte and Eastern gamagrass tripsacum. The author crossed teosinte and tripsacum and found that the progeny bore traits (inherited from tripsacum) that have been considered to be missing links in the origin of maize. In another study, selection during maize domestication was found to influence plant morphology. The gene responsible for the morphology difference between maize (single, large stalks) and teosinte (multiple, thin stalks) plants is *teosinte branched1*. This gene is similar in maize and teosinte, but the gene regulatory region differs between these plants, and therefore it is thought to influence the morphological differences between maize and teosinte (Wang et al 1999). While these new findings increase understanding of the genetic differences between maize and teosinte, questions about the relationship between them still remain.

Whatever the genetic relationship between teosinte and maize, much of their importance in society and science stems from the fact that both plants produce starch. Starch is a semi-crystalline glucose polymer that serves as an energy storage material in plants, and is the most important carbohydrate source in the human diet. Many previous studies have examined the genetic and morphological similarities and differences between teosinte and maize plants, but few studies have determined differences in their economically important product, starch. This study was undertaken to characterize the structural and chemical properties of starch isolated from modern maize, Chalco teosinte and a Chalco teosinte-maize cross. Any unique and desirable characteristics of teosinte starch could be introduced into maize starch to increase the diversity of starch structures and properties and add value to maize starch by selective crossing the two plants.

Materials and Methods

BSSS maize seeds were grown in Ames, Iowa, 1990 and BSSS-Teo Mix seeds were grown in Ames, Iowa, 1994. BSSS-Teo Mix is a Chalco teosinte (cornbelt adopted for photo sensitivity) and BSSS maize cross, created in Iowa State University Agronomy Department and randomly mated for several generations. Teosinte seeds were provided by the North Central Regional Plant Introduction Station (Ames, IA). Teosinte race Chalco was grown in Federal District, Mexico, collected in March of 1988, and teosinte race Zea Luxuraians was grown in Juliapa, Guatemala, collected in December of 1975. Teosinte race Central Plateau was a hybrid swarm associated with hybrid corn and sorghum. The second and third backcrosses used in this study were grown in Michoacan, Mexico, collected in November of 1992. Commercial normal, or common, maize starch was provided by Cerestar USA, Inc. (Hammond, IN, USA). Isoamylase (EC 3.2.1.68) from *Pseudomonas amyloderamosa* was purchased from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). Amyloglucosidase (EC 3.2.13) from Rhizopus mold was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sepharose CL-2B gel was purchased from Pharmacia, Inc. (Piscataway, NJ, USA). Other chemicals were reagent grade and were used without further purification.

Starch Isolation

Starches were isolated from BSSS maize and BSSS-Teo Mix according to a method adopted from Badenhuizen (1964), Kasemsuwan et al (1995), and Watson (1955). Clean, whole seeds were steeped in a sodium meta-bisulfite ($Na_2S_2O_5$) aqueous solution (0.30%) at ambient temperature for 24 hrs then ground on high speed in a Waring Blendor (Waring Consumer Products, East Windsor, NJ, USA) (3 min) and filtered through a 30 μm nylon screen. The fiber fraction was collected, ground (3 min), and filtered through the same nylon screen. Starch was

collected by centrifugation (Sorvall RC-5C, Kendro Laboratory Products, Newtown, CT, USA) (6,600 *g*, 20 min), and the yellow protein layer was removed. This procedure was repeated until no yellow protein appeared. The isolated starch was then suspended in a 0.1 M-NaCl solution with 10% volume of toluene, stirred for at least 1 hr, and allowed to stand until the starch settled. The protein-toluene layer was siphoned off and this procedure was repeated until the toluene layer was clean. The remaining starch was washed with distilled water (3 times) and absolute ethanol (2 times). Starch was collected by filtration on a Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England), rinsed with ethanol, and dried at 32°C for 48 hrs.

Chalco teosinte seeds were soaked in the 0.30% $Na_2S_2O_5$ solution at ambient temperature for 24 hrs and ground (20 sec) to remove the stiff outer seed coats, then soaked in fresh 0.30% $Na_2S_2O_5$ for 18 hrs. Broken seeds were ground (3 min) and washed through a 30 μm nylon screen. The remaining fiber fraction was ground and washed through the same screen three times, then placed in 0.80% $Na_2S_2O_5$ and soaked at 4°C for 5 days. After soaking, the fiber was ground 5 times (2 min each) and washed through a 30 μm screen. The resulting fiber fraction was ground 4 times (2 min each) and washed through the same 30 μm screen. Protein removal was accomplished by toluene washes as described above. Centrifugation could not be used to physically separate protein and starch because the Chalco teosinte starch pellet was very easily disrupted and some starch remained in the supernatant even after long periods of centrifugation. Therefore, all protein was removed by toluene washes. Starch was washed and collected as described above. One batch of starch was isolated from each starch source due to limited sample quantities.

Starch Content

Starch content was determined according to a method adopted from Perrera et al (2001) and Umemoto et al (1995). Whole seeds (~100 mg) were soaked in 10 mL of distilled water at 4°C for at least 80 hrs and ground with a mortar and pestle. Water was added as needed to grind the endosperm and the slurry was centrifuged (15,000 *g*, 50 min); supernatant was removed, and stored at 4°C. Dimethyl sulfoxide (DMSO) (10 mL) was added to the pellet and heated in a boiling water bath with stirring for 1 hr, then stirred at room temperature for 48 hrs. Starch-containing DMSO solution (1 mL) was removed (no hulls), and 5.0 mL of absolute ethanol was added. Starch was collected by centrifugation (15,000 *g*, 25 min) and the supernatant was discarded. Distilled water (10 mL) was added to the starch pellet, heated with stirring for 20 min in a boiling water bath, and allowed to cool. The supernatant collected after grinding kernels was removed from 4°C storage, heated in a boiling water bath with stirring for 40 min, cooled to room temperature, and the volume was recorded. From this point on, supernatant and DMSO-treated solutions were treated identically. An amyloglucosidase (AMG) solution (activity 80 units/mL) was prepared in 0.1 M sodium acetate buffer, pH 4.5. Sample (300 µL) and AMG solution (100 µL) were combined and held at 55°C for 1 hour, then centrifuged at 10,000 *g* for 10 min. A blank containing water in place of sample and was treated identically to samples. Non-enzyme treated controls were prepared from 300 µL of sample and 100 µL water. All samples and controls were analyzed for glucose content using a Sigma Glucose Diagnostic Kit (Sigma Chemical Co., St. Louis, MO, USA). Glucose contents of the controls were subtracted from the glucose contents of the enzyme treated samples and the total amount of starch-derived glucose in each sample was calculated.

Protein Content

Protein contents of BSSS maize, BSSS-Teo Mix, Chalco, Zea Luxurians, and Central Plateau teosinte seeds were determined in the Grain Quality Laboratory at Iowa State University. Whole kernels (approximately 2.0 g) were ground using an MC200 Miracle Mill (Miracle Exclusives, Inc., Port Washington, NY, USA) and analyzed by using a nitrogen analyzer (PE 2410 Series II, Perkin Elmer, Norwalk, CT, USA). A protein conversion factor of 6.25 was used for all calculations.

Starch Morphology

Morphology of starch granules was studied by scanning electron microscopy in the Bessey Microscopy Facility at Iowa State University. Starches were mounted on brass disks with double-sided sticky tape, coated with gold and palladium (60:40), and observed using a scanning electron microscope (JEOL model 1850, Tokyo, Japan).

X-Ray Diffraction

Starch samples (0.7 g) were equilibrated in a 100% relative humidity chamber at 25°C for 24 hrs. X-ray diffraction patterns were obtained using copper, graphite monochrometer filtered, Kα radiation with a Siemens D-500 diffractometer (Madison, WI, USA). Starches were scanned from 4° to 37° with a 0.05° step size and a count time of 3 sec.

Molecular Weight

Starch samples were prepared for gel permeation chromatography (GPC) according to the method of Perera et al (2001). Two milliliters of water (containing 6 mg of starch) was injected onto a column (0.75 ID x 45 cm) packed with Sepharose CL-2B gel. An eluent solution containing 1 mM NaOH and 25 mM NaCl was used at a flow rate of 0.5 mL/min. Fractions were collected at 2.5 min intervals and analyzed for total carbohydrate content (Dubois et al

1956) using a test tube-scale analysis (1.4 mL total volume), absorbance was analyzed in an ultra-microplate reader (Bio-Tek Instruments, Winooski, VT, USA). Blue value was analyzed in microplates using chemical reaction reported by Jane and Chen (1992).

Amylopectin molecular weight was determined by high-performance size-exclusion chromatography (HPSEC) equipped with multiangle laser light scattering (MALLS, model Dawn-F, Wyatt Tech. Co., Santa Barbara, CA, USA) and refractive index (RI) (HP1047A, Hewlett Packard, Wilmington, DE, USA) detectors. Shodex OH pak KB-G guard column and KB-806 and KB-804 analytical columns (Shodex Denko, Tokyo, Japan) were used for sample separation. The columns and injector were maintained at 55°C using a CH-460 column heater and a TC-50 controller (Eppendorf, Madison, WI, USA). The temperature of the RI detector was set at 30°C. Distilled, deionized, and degassed water (18.2 MΩ cm) was used as the mobile phase, flow rate 0.6 mL/min (HP1050 series isocratic pump, Hewlett Packard, Wilmington, DE), following the method reported by McPherson and Jane (2000).

Iodine Affinity and Amylose Content

Iodine affinities of defatted whole starch samples were determined using a potentiometric autotitrator equipped with Meterodata recording software (702 SM Titrino, Brinkman Instrument, Westbury, NY, USA). Apparent amylose content was determined by the method of Schoch (1964).

Thermal Properties of Starch

Thermal properties of starches were determined by using a differential scanning calorimeter (DSC) equipped with an Intracooler II system and Pyris thermal analysis software (DSC-7, Perkin-Elmer, Norwalk, CT, USA). Starch (2 mg, dsb) and water (6 μL) were sealed in an aluminum pan and equilibrated for at least 1 hr. Samples were scanned from 10 to 100°C at a

heating rate of 10°C/min using an empty pan as a reference and indium as a standard. Enthalpy change (ΔH), onset temperature (T_o), peak temperature (T_p), and conclusion temperature (T_c) were calculated automatically. Analysis of retrograded starches followed the same method after the gelatinized samples were stored at 4°C for 7 days.

Pasting Properties of Starch

Pasting profiles of starches were determined using a Rapid Visco Analyzer (RVA-4, Newport Scientific, Warriewood, Australia). Starch suspensions (8% dsb) were equilibrated at 50°C for 1 minute, heated to 95°C at 6°C/min, held at 95°C for 4 min, and cooled down to 50°C at 6°C. Stirring rate was 160 rpm throughout the experiment.

Branch Chain Length Distribution of Starch

Whole starch was debranched using isoamylase according to the method of Jane and Chen (1992). Branch chain lengths were determined using a high-performance anion exchange chromatograph with a postcolumn amyloglucosidase reactor and a pulsed amperometric detector (HPAEC-ENZ-PAD) (Wong and Jane 1997). Samples were separated using a PA-100 guard column and PA-100 anion exchange analytical column (Dionex, Sunnyvale, CA, USA). The mobile phase used for separation consisted of two eluents, degassed with helium by a Dionex gas module, at a flow rate of 0.5 mL/min. Eluent A (100 mM NaOH), and eluent B (100 mM NaOH and 300 mM $NaNO_3$) were used in a separation gradient of 99% A and 1% B for 0 to 5 min with the proportion of eluent B increasing linearly to 8% for 5 to 30 min. The proportion of eluent B increased linearly to 30% for 30 to 150 min, then to 45% for 150-200 min. Histograms represent quantitative detector response to glucose derived from chains of a particular chain length, and not the number of branch chains at that chain length.

Experimental Design and Statistical Analysis

Starch thermal properties (determined by DSC) were analyzed in triplicate. Amylopectin molecular weight (determined by HPSEC and GPC), starch branch chain length distribution (determined by HPAEC-ENZ-PAD), pasting properties (determined by RVA), and amylose contents (determined by iodine affinity) were analyzed in duplicate. One sample was analyzed by scanning electron microscopy and X-ray diffraction. Statistical analysis (ANOVA) of DSC data was performed using Excel (Microsoft Co., Redmond, WA, USA) with P=0.05.

Results and Discussion

Protein and starch contents of maize and teosinte seeds are presented in Table 1. All seeds varied in protein content from 6.7% to 13.6%. BSSS maize, BSSS-Teo Mix, and Zea Luxurains teosinte seeds had relatively similar proportions of protein (10.3 to 13.6%), while Chalco and Central Plateau teosinte seeds contained approximately half as much protein (approximately 7%). Starch contents of BSSS maize and BSSS-Teo Mix seeds (71.3 to 72.9%) were notably larger than teosinte seed starch contents (25.7 to 27.0%). Liao and Konlande (1973) found that the Guerrero variety of teosinte seeds contained 12.0% protein and 60.9% available carbohydrate (total carbohydrate – fiber). The protein content reported previously by Liao and Konlande is comparable to the protein content of Zea Luxurians teosinte seeds analyzed in this study, but the starch and available carbohydrates varied widely.

Differences in starch and protein contents between maize and teosinte seeds were expected, considering the differences in seed morphology. Maize seeds (BSSS maize, and BSSS-Teo Mix) were yellow colored, large, and generally spherical with very thin, pliable outer seed coats. Teosinte seeds, however, were dark brown or gray with speckles, small and triangular in

Table 1. Protein and starch contents (% db) of whole seeds

Sample	Protein Content[a]	Starch Content[b]
BSSS Maize	10.3 ± 0.4	72.9 ± 1.4
BSSS-Teo Mix	13.6 ± 1.1	71.0 ± 1.0
Chalco Teosinte	6.7 ± 0.1	27.0 ± 0.7
Central Plateau Teosinte	7.3 ± 0.7	26.2 ± 0.1
Zea Luxurains Teosinte	13.2 ± 0.8	25.7 ± 0.9

[a]Kernel protein content determined by nitrogen analyzer. Values are means of two determinations, mean ± standard deviation.
[b]Starch content determined by an enzymatic method. Values are means of two determinations, mean ± standard deviation.

shape, and had very thick, brittle outer seed coats. The morphological differences between teosinte fruit spikes and maize ears, and differences in seed morphologies have created much interest and debate about the relationship between teosinte and maize (Mangelsdorf 1950, Robson et al 1976, Beadle 1980, Iltis 1983, Dorweiler and Doebley 1997).

Starch granule morphologies, determined by scanning electron micrographs, are shown in Figure 1. BSSS maize (Fig. 1A, B) starch granules demonstrated spherical and angular shapes common to normal maize starch (Perrera et al 2001) with the exception of some dimpled granules. BSSS-Teo Mix starch granules (Fig. 1C, D) were generally similar to BSSS maize starch granules, but there were more dimpled granules and some granules had broken or cracked surfaces. Teosinte starch granules (Fig. 1E, F) were spherical, angular, dimpled, broken, or hollow. Higher magnification of Chalco teosinte starch granules (Fig. 1G, H) shows that some granules were hollow or contained holes. These hollow starch granules may have contributed to the need for starch extraction and purification procedures different from those used for maize starch. Even with these differences between Chalco teosinte starch and maize starch granules,

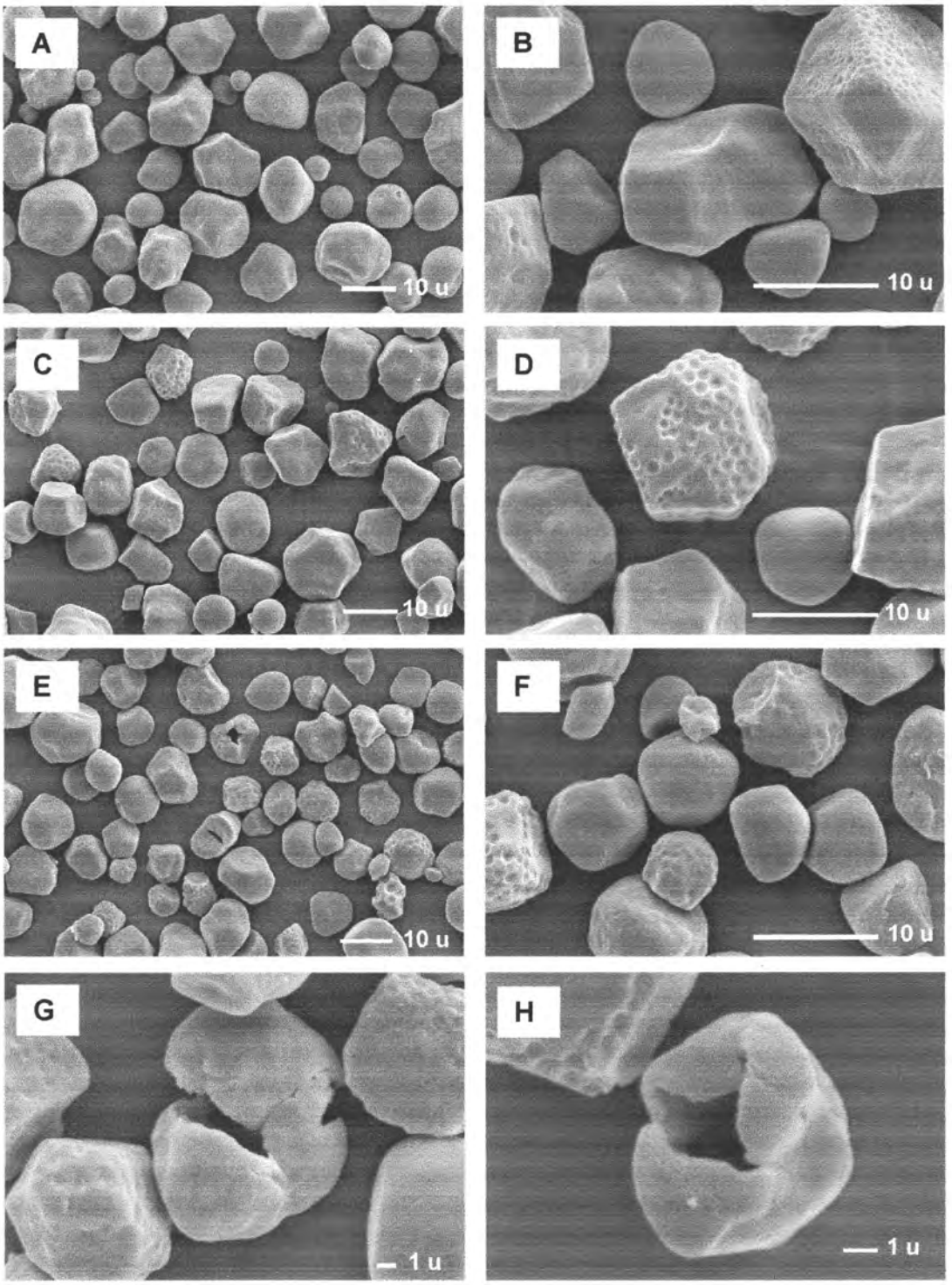

Figure 1. Scanning electron micrographs of BSSS maize (A, B), BSSS-Teo Mix (C, D), and Chalco teosinte (E, F, G, H) starch granules.

Chalco teosinte starch granule morphologies were more similar to maize starches than to other grain, root, tuber, or bean starch granules (Jane et al 1994).

X-ray diffraction patterns of starches are shown in Figure 2. All starches demonstrated typical A-type X-ray diffraction patterns (Zobel 1964). X-ray diffraction patterns indicated relatively similar degrees of crystallinity for all starches; crystallinity was calculated as (area in crystalline peaks)/(total area)*100. Degrees of crystallinity were 38, 40, 46, and 40% for commercial normal maize, BSSS maize, BSSS-Teo Mix, Chalco teosinte starches, respectively.

Amylopectin weight average molecular weights measured by HPSEC were similar for all samples, ranging from 7.2 to 7.5 x 10^8. Results are given in Table 2. BSSS Maize amylopectin had the smallest molecular weight (7.2 x 10^8) while BSSS-Teo Mix amylopectin had the largest (7.5 x 10^8). The radii of gyration for amylopectins followed in the same pattern with BSSS-Teo Mix amylopectin having the largest gyration radius and (318.0 nm) and BSSS maize amylopectin having the smallest (311.1 nm).

Table 2. Molecular weighs and gyration radii of amylopectin from starches

Sample	Amylopectin Weight Ave M_w (M_w x 10^8)[a]	Gyration Radius of Amylopectin (R_z) (nm)[b]
BSSS Maize	7.2 ± 0.1	311.1 ± 3.9
BSSS-Teo Mix	7.5 ± 0.02	318.0 ± 2.0
Chalco Teosinte	7.4 ± 0.06	314.9 ± 2.0

[a]Molecular weight determined by light scattering and refractive index detectors. Values are means of two determinations, mean ± standard deviation.
[b]Gyration radii determined by light scattering and refractive index detectors. Values are means of two determinations, mean ± standard deviation.

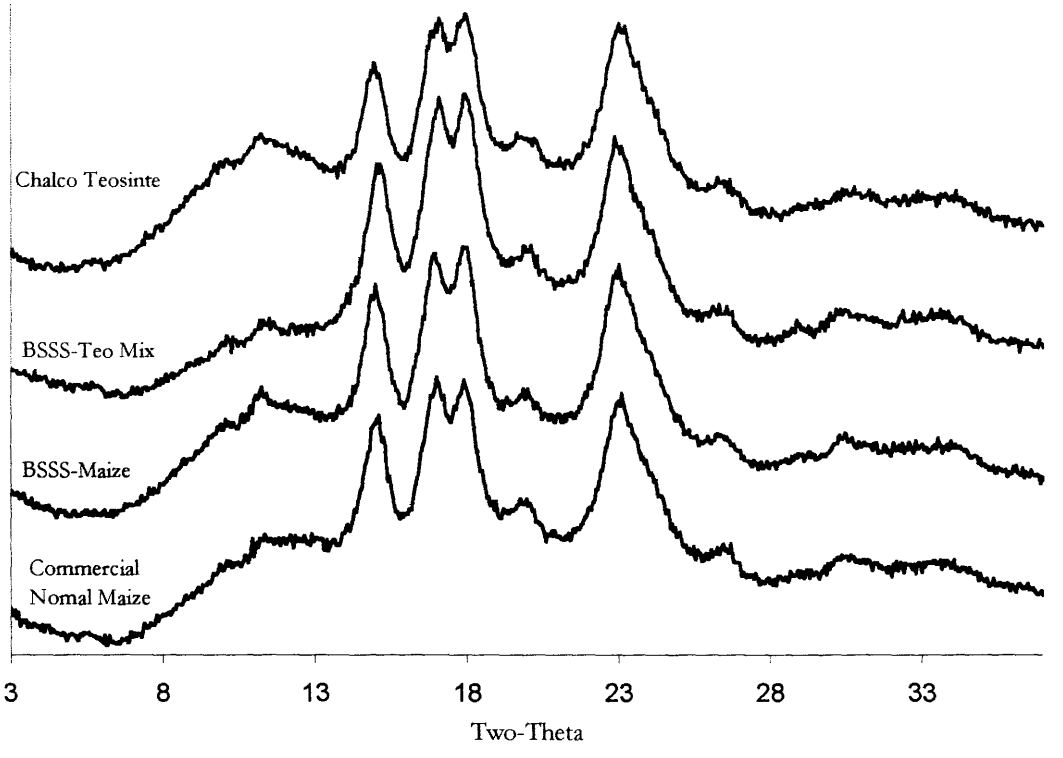

Figure 2. X-Ray diffraction patterns of starches

Molecular size distributions determined by gel permeation chromatography (GPC) are presented in Figure 3. The first peak in each chromatogram corresponds to amylopectin and the second peak corresponds to amylose. Chromatograms from all starches displayed these two peaks. Areas under the amylopectin and amylose peaks (total carbohydrate profile) were used to calculate the amylose contents of the starches.

Starch iodine affinities and amylose contents determined by iodine titration and amylose contents determined by GPC are shown in Table 3. Starch iodine affinities ranged from 5.16 (BSSS maize) to 5.62 (Chalco teosinte). Amylose contents were 27.14 and 29.61% for BSSS maize and Chalco teosinte starches, respectively. The amylose contents determined by GPC were generally in agreement with the amylose contents determined by iodine titration. All starches used in this study contained 27 to 29% amylose (determined by iodine titration), which is the proportion of amylose expected for normal maize starch.

The branch chain length distributions of commercial normal maize, BSSS maize, BSSS-Teo Mix, and Chalco teosinte starches are shown in Figure 4 and the accompanying data are given in Table 4. Commercial normal maize, BSSS-Teo Mix, and Chalco teosinte starches showed the first peak at branch chain length 13 dp; BSSS maize starch showed the first peak at 14 dp. The second peak appeared at branch chain length of 45 dp for commercial normal and BSSS maize starches, 46 dp for BSSS-Teo Mix starch, and 44 dp for Chalco teosinte starch. The average branch chain length was 25.3, 25.1, 24.7, and 23.5 dp for commercial normal maize, BSSS maize, BSSS-Teo Mix, and Chalco teosine starches, respectively. The distribution proportions were similar for commercial normal maize and BSSS maize starch. Commercial normal maize starch had the lowest proportion of branches 13-24 dp (46.0%) while Chalco teosinte starch had the largest (48.2%). Chalco teosinte starch also demonstrated the smallest

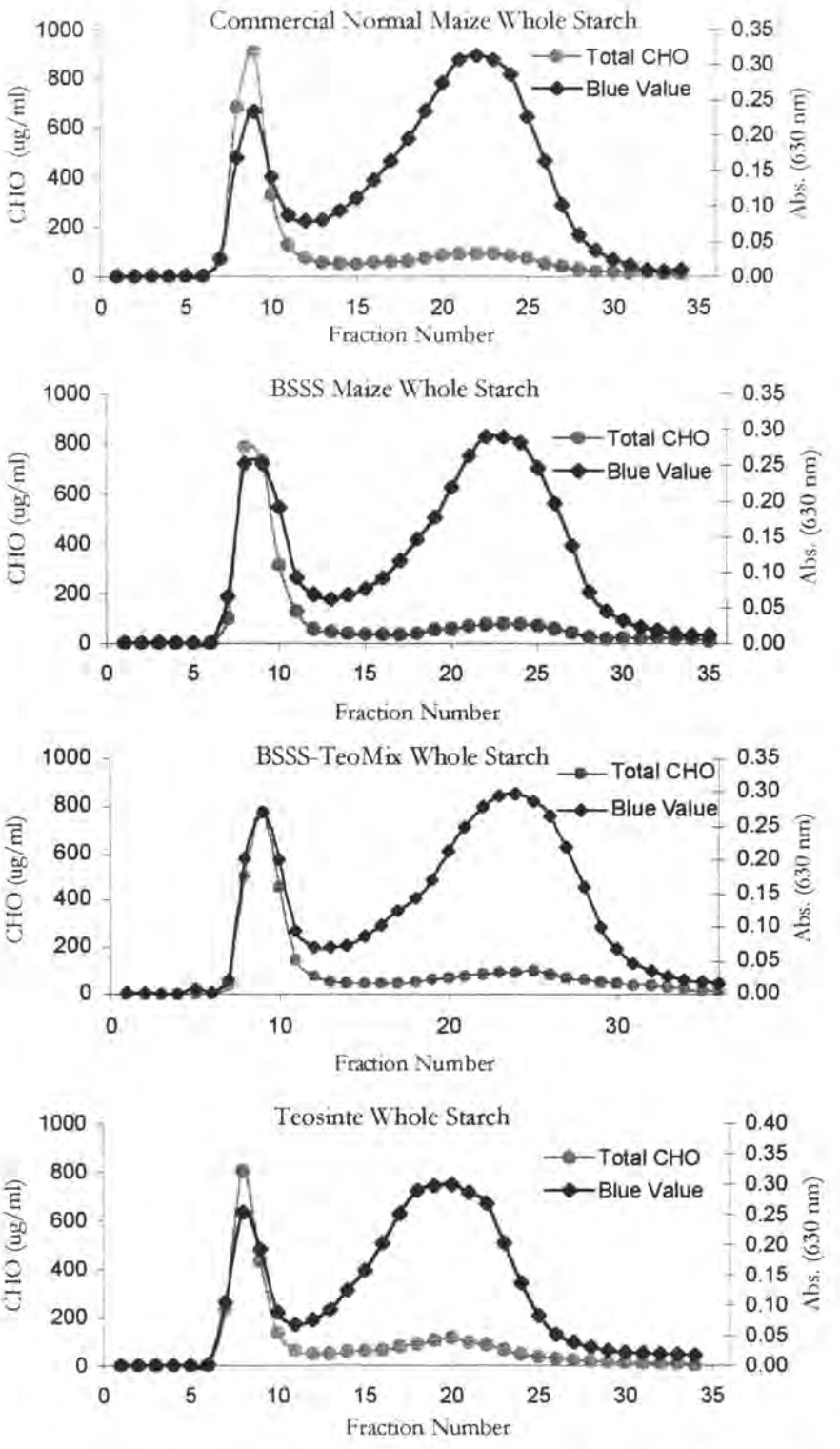

Figure 3. Gel permeation chromatography profiles of starches determined using Sepharose CL-2B gel. Chromatograms are typical of each starch sample.

Table 3. Amylose contents of maize, maize-teosinte cross and teosinte starches determined by autotitration and gel permeation chromatography

	Iodine Titration[a]		Gel Permeation Chromatography[c]
Sample	**Iodine Affinity**	**Amylose Content (%)[b]**	**Amylose Content (%)**
Commercial Normal Maize	5.33 ± 0.04	28.04 ± 0.21	30.88 ± 1.31
BSSS Maize	5.16 ± 0.25	27.14 ± 1.30	26.15 ± 0.41
BSSS-Teo Mix	5.32 ± 0.24	28.02 ± 1.28	32.80 ± 1.04
Chalco Teosinte	5.62 ± 0.02	29.61 ± 0.11	29.75 ± 1.05

[a]Values are means of three determinations, mean ± standard deviation.

[b]Apparent amylose content determined by dividing iodine affinity by 0.19.

[c]Amylose content = (area under amylose peak/total area) x 100. Values are means of two determinations, mean ± standard deviation.

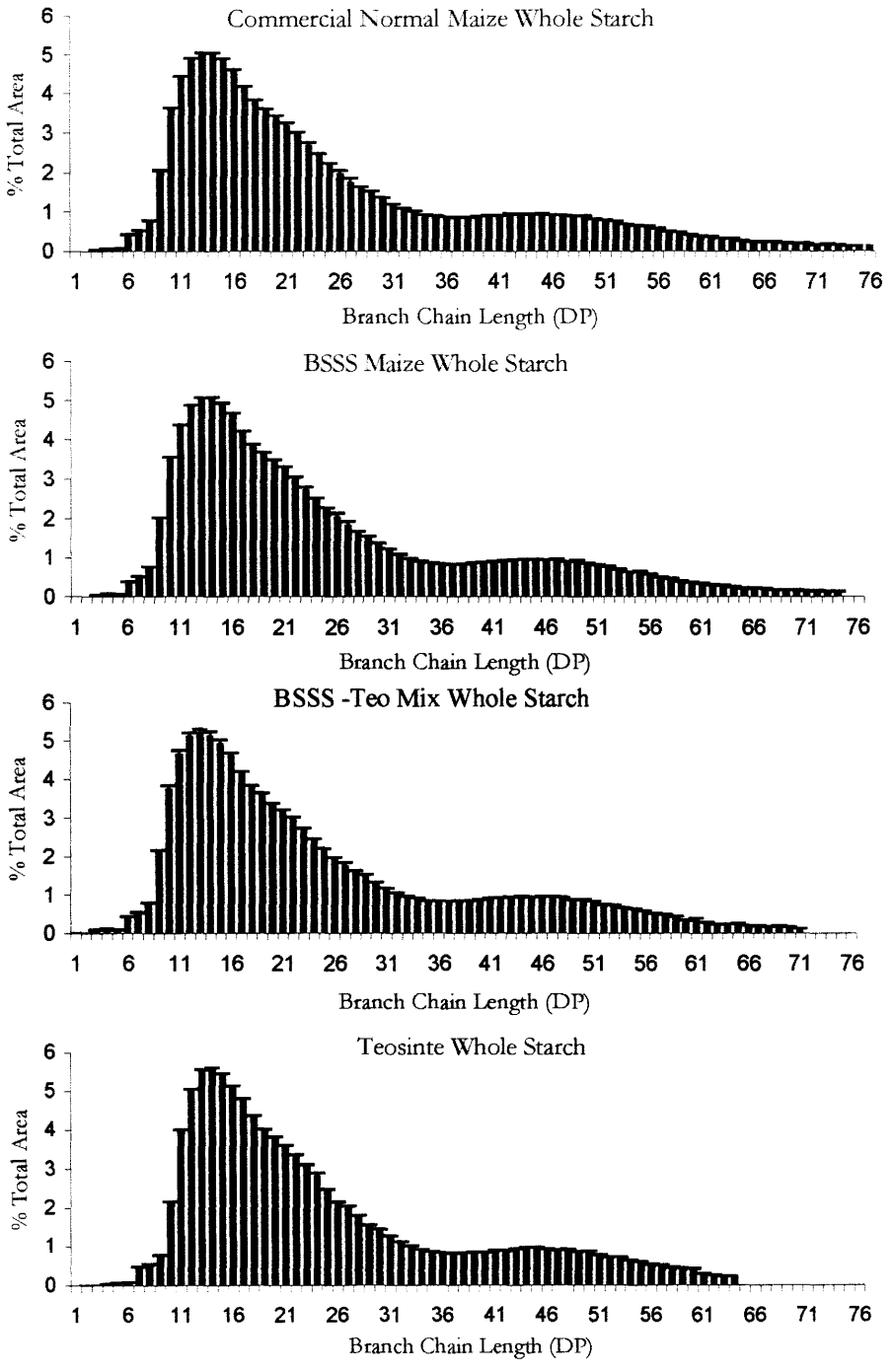

Figure 4. Branch chain length distributions of whole starches determined by a high-performance anion exchange chromatograph equipped with a postcolumn amyloglucosidase reactor and pulsed amperometric detector (HPAEC-ENZ-PAD). Histograms are averages of two runs.

Table 4. Branch-chain-length distributions of maize, maize-teosinte cross and teosinte whole starches[a]

Sample	Peak 1	Peak 2	Average DP	% Distribution (DP)				Highest Detectable DP
				3-12	13-24	25-36	≥ 37	
Commercial Normal Maize	13	45	25.3 ± 0.4	16.8 ± 0.5	45.5 ± 0.9	16.3 ± 0.4	21.4 ± 0.9	76
BSSS Maize	14	45	25.1 ± 0.3	16.6 ± 0.1	46.0 ± 0.6	16.5 ± 0.4	20.9 ± 0.9	74
BSSS-Teo Mix	13	46	24.7 ± 0.4	17.6 ± 0.5	46.0 ± 0.5	16.0 ± 0.4	20.4 ± 0.8	71
Chalco Teosinte	13	44	23.5 ± 0.2	18.5 ± 0.3	48.2 ± 0.3	15.6 ± 0.2	17.9 ± 0.5	63

[a]Determined by high-performance anion exchange chromatography with postcolumn amyloglucosidase reactor and pulsed amperometric detector (HPAEC-ENZ-PAD). Values are means of two determinations, mean ± standard deviation. DP = degree of polymerization.

proportion of branches of DP greater than 37 (17.9%). Overall, the branch chain length distribution of Chalco teosinte starch was similar to branch chain length distributions of maize starches, and different from wheat, barley, and rice starch branch chain length distributions (Jane et al 1999).

Table 5. Gelatinization properties of starches[a]

Sample	T_o (°C)[b]	T_p (°C)[c]	T_c (°C)[d]	ΔH (J/g)[e]
Commercial Normal Maize	67.8 ± 0.2	71.6 ± 0.1	75.2 ± 0.1	12.9 ± 0.4
BSSS Maize	66.1 ± 0.1	70.7 ± 0.1	75.6 ± 0.2	15.2 ± 0.4
BSSS-Teo Mix	64.0 ± 0.2	69.1 ± 0.7	74.3 ± 0.3	13.9 ± 1.3
Chalco Teosinte	61.4 ± 0.3	68.0 ± 0.2	74.2 ± 0.2	11.4 ± 1.4

[a] T_o T_p T_c = onset, peak, and conclusion temperatures of endotherm determined by differential scanning calorimetry. ΔH = enthalpy change of gelatinization. Values are means of three determinations, mean ± standard deviation.
[b] All samples are significantly different (p = 0.05).
[c] All samples are significantly different (p = 0.05).
[d] Commercial normal maize starch is significantly different from BSSS-Teo Mix and Chalco teosinte starches. BSSS maize starch is significantly different from BSSS-Teo Mix, and Chalco teosinte starches (p = 0.05).
[e] BSSS maize starch is significantly different from commercial normal maize and Chalco teosinte starches (p = 0.05).

Gelatinization properties of maize and teosinte starches were determined by using DSC, and results are given in Table 5. Onset gelatinization temperatures were 67.8, 66.1, 64.0, and 61.4°C for commercial normal maize, BSSS maize, BSSS-Teo Mix, and Chalco teosinte starches, respectively. The lower onset gelatinization temperature of Chalco teosinte starch (61.4°C) and BSSS-Teo Mix starch (64.0°C) are attributed to the smaller proportion of long branch chains (dp>37), 17.9% and 20.4%, respectively, and more short chains (3-12 dp), 18.5% and 17.6%, respectively. The range of temperature from gelatinization onset (T_o) to completion (T_c) was

relatively small for commercial normal maize starch (7.4°C) and larger for Chalco teosinte starch (12.8°C). The gelatinization temperature ranges of BSSS-Teo Mix (10.3°C) and BSSS maize (9.5°C) starches were between commercial normal maize and Chalco teosinte starches. The wider gelatinization temperature range of Chalco teosinte starch may have resulted from the damaged granule structures revealed by the SEM micrographs of the starch granules shown in Figure 1. Enthalpy change during gelatinization (ΔH) was the smallest for Chalco teosinte starch and the largest for BSSS maize starch, with commercial normal maize and BSSS-Teo Mix starches in between.

Table 6. Retrogradation properties of starches[a]

Sample	T_o (°C)[b]	T_p (°C)	T_c (°C)	ΔH (J/g)	Retrogradation (%)[c]
Commercial Normal Maize	37.1 ± 0.8	49.1 ± 1.1	61.1 ± 0.3	6.0 ± 0.2	46.4 ± 2.3
BSSS Maize	39.3 ± 1.4	50.3 ± 0.5	61.8 ± 0.3	6.2 ± 0.6	41.0 ± 3.8
BSSS-Teo Mix	39.6 ± 0.6	50.2 ± 0.6	62.1 ± 0.6	6.9 ± 0.1	49.7 ± 5.6
Chalco Teosinte	40.1 ± 0.3	50.3 ± 0.7	61.1 ± 0.2	6.4 ± 0.8	56.2 ± 3.7

[a] T_o T_p T_c = onset, peak, and conclusion temperatures of endotherm determined by differential scanning calorimetry. ΔH=enthalpy change of gelatinization. Values are means of three determinations, mean ± standard deviation.
[b] Commercial normal maize starch is significantly different from BSSS-Teo Mix and Chalco teosinte starches ($p = 0.05$).
[c] % retrogradation = (enthalpy change of retrograded starch/enthalpy change of native starch) *100.

Properties of retrograded starch were determined by using DSC after gelatinized samples were stored at 4°C for 7 days; results are given in Table 6. All retrograded starch samples showed lower peak dissociation, or melting, temperatures, wider temperature ranges, and lower enthalpies of melting. Although Chalco teosinte starch had the smallest enthalpy change of

gelatinization, enthalpy of melting for retrograded Chalco teosinte starch was comparable to the other starches. Therefore, Chalco teosinte starch was calculated to have a larger degree of retrogradation, 56.2%, whereas the other starches demonstrated 46.4, 41.0, and 49.7% retrogadation for commercial normal maize, BSSS maize, and BSSS-Teo Mix starches, respectively.

Pasting profiles of the maize and Chalco teosinte starches are shown in Figure 5, and the accompanying viscosity data are given in Table 7. BSSS-Teo Mix and BSSS maize starches showed the lowest pasting temperatures (72.2 and 71.3°C) and the largest peak viscosities (174.6 and 187.3 RVU, respectively). The commercial normal maize and Chalco teosinte starches demonstrated larger pasting temperatures (83.4 and 84.7°C) and smaller peak viscosities (158.9 and 135.8 RVU, respectively) when compared with BSSS maize and BSSS-Teo Mix starches. Chalco teosinte starch showed the largest breakdown, or shear thinning (79.0 RVU), followed by the smallest final viscosity (124.6 RVU) and smallest setback (67.5 RVU) of all starches in this study. The severity of shear thinning found in teosinte starch was comparable to that observed in waxy maize starch (Perera et al 2001). The viscosities at the trough (lowest point) in the amylograms were 41.8 and 38.2% of the peak for Chalco teosinte and waxy maize starches, respectively. The commercial normal maize, BSSS maize, and BSSS-Teo Mix starches demonstrated 63.1, 57.8, and 60.0% peak viscosity at the trough. The drastic effects of shear thinning found in waxy maize starch have been attributed to the lack of amylose, which is thought to maintain granular integrity. However, lack of amylose is not the cause of this phenomenon in Chalco teosinte starch because both GPC and iodine titration showed that teosinte starch contains approximately 30% amylose. Branch chain length distributions of these starches demonstrated that Chalco teosinte starch had shorter branch chain lengths than the

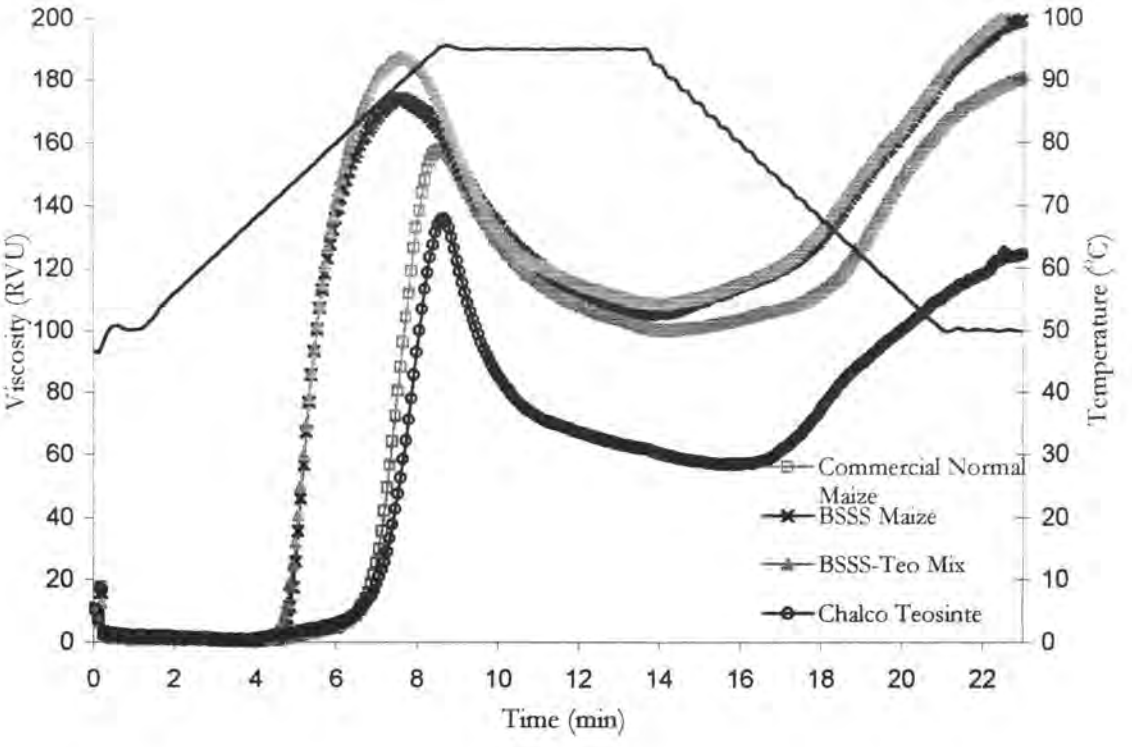

Figure 5. Starch pasting properties analyzed by using a Rapid Visco Analyzer (8% dsb). Profiles are averages of two determinations.

Table 7. Pasting profiles of maize, maize-teosinte cross and teosinte starches[a]

Sample	Peak 1	Breakdown	Final Viscosity	Setback	Peak Time[b]	Pasting Temp (°C)
Commercial Normal Maize	158.9 ± 5.6	58.7 ± 8.0	180.3 ± 5.0	80.6 ± 7.7	8.6 ± 0.05	83.4 ± 0.3
BSSS Maize	174.6 ± 8.3	69.8 ± 3.5	199.6 ± 7.1	94.9 ± 4.8	7.6 ± 0.10	72.2 ± 0.3
BSSS-Teo Mix	187.3 ± 5.4	79.0 ± 2.5	203.7 ± 2.9	95.3 ± 4.9	7.5 ± 0.00	71.3 ± 0.4
Chalco Teosinte	135.8 ± 1.7	79.0 ± 3.3	124.6 ± 2.5	67.5 ± 4.7	8.6 ± 0.05	84.7 ± 0.5

[a]Determined by using a Rapid Visco Analyzer measuring in RVU. Values are means of two determinations, mean ± standard deviation.

[b]Peak time determined in minutes.

other starches, and the lack of very long branch chains may have been responsible for the large shear thinning of Chalco teosinte starch. Overall, the Chalco teosinte starch pasting profile was similar to that of commercial normal maize starch, and different from previously reported pasting profiles of wheat, barley, and rice starches (Jane et al 1999).

Conclusions

This study showed that teosinte and maize seeds differ in starch and protein contents, with maize seeds having a much greater proportion of starch than teosinte seeds. Starch isolated from Chalco teosinte, BSSS maize, and BSSS-Teo Mix was characterized and compared with a commercial normal maize starch. Starch granule morphology, determined by SEM, demonstrated that some Chalco teosinte starch granules were irregularly shaped, broken, or hollow, but were generally similar to commercial normal maize, BSSS maize, and BSSS-Teo Mix starch granules. X-ray diffraction showed that all four of these starches were A-type starches with only slight differences in diffraction intensities, and molecular weights of amylopectins (determined by HPSEC and GPC) were generally comparable. The amylose contents of starches (determined by GPC and iodine titration) were also comparable. The major differences between these four starches were observed in branch chain length distributions, thermal and pasting properties.

Chalco teosinte starch had a lower onset gelatinization temperature (determined by DSC) and lower enthalpy of gelatinization as compared with the maize starches, and the retrogradation rate for this starch was relatively high. Pasting profiles, determined by RVA, showed that BSSS maize and BSSS-Teo Mix had relatively large peak viscosities, but Chalco teosinte starch had a relatively small peak viscosity and a large breakdown. Interestingly, BSSS-Teo Mix demonstrated

the largest peak viscosity. Branch chain length distributions showed that all four starches had similar branch chain length distribution profiles, but Chalco teosinte starch had the shortest long branch chains and average chain length, and BSSS-Teo Mix had the second shortest average chain length. In general, Chalco teosinte starch was similar to the maize starches, and comparisons with previously published studies showed that Chalco teosinte starch was more similar to maize starch than to other major cereal starches.

Acknowledgments

We thank Dr. Kit-Sum Wong for her generous technical support, the Iowa Corn Promotion Board for providing funds, and the North Central Regional Plant Introduction Station for providing teosinte seeds. We would also like to thank the ISU Bessey Microscopy Facility and Dr. Scott Schlorholtz for their help with the SEM micrographs and X-ray diffraction patterns, respectively.

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CRYSTALLINITY AND MELTING PROPERTIES OF A STARCH MODEL SYSTEM

Introduction

Two methods for determination of crystallinity in semi-crystalline materials have been adopted from other research areas and used heavily in starch research. Wakelin et al (1959) created a model system for determination of crystallinity in cotton samples, and Hermans and Weidinger (1961) calculated degree crystallinity of polyethylene samples by comparing area of amorphous backgrounds to crystalline peaks. Since then, much work has been done to develop accurate methods of starch crystallinity determination (Cairns et al 1997, Gernat et al 1990, Paris et al 1999, Polizzi et al 1990). Other methods have been developed to determine relative crystallinity of starch samples (Demeke et al 1999, Forssell et al 1999).

Differential scanning calorimetry (DSC)-determined enthalpy change of starch gelatinization can theoretically reflect the degree of crystallinity in a starch sample (Ahmed and Leliéver 1978). Some authors have made comparisons between X-ray diffraction-determined degree of crystallinity and DSC-determined enthalpy change of melting (Leliéver, 1975), but little work has been done to determine the exact relationship between degree of crystallinity and enthalpy of melting in starch samples. The goal of this study was to develop a relationship between crystallinity and enthalpy change of melting in a starch model system. Normal maize starch Naegeli dextrin and gelatinized, amorphous waxy maize starch were used as the crystalline and amorphous standards, respectively. Individual components and mixtures thereof were analyzed by X-ray diffraction and DSC and trends in behavior were determined.

Materials and Methods

Amorphous Waxy Maize Starch

Three grams of waxy maize starch was wetted with 30 mL of distilled water and dispersed in 270 mL Dimethylsulfoxide (DMSO). The starch-DMSO solution was heated in a boiling water bath with stirring for 1 hr and stirred at room temperature for 24 hrs. Amorphous starch was precipitated with 3 times volume (900 mL) of 100% methanol and collected by filtration on a Whatman No. 5 filter paper (Whatman International, Ltd., Maidstone, England). Starch was resuspended in 250 mL of 100% methanol, stirred vigorously for 30 min, and collected by filtration. This procedure was repeated 4 times, until a fine white powder resulted. Amorphous starch was dried for 24 hrs at 32°C, weighed into 4 separate samples (0.700, 0.525, 0.350, and 0.175 g), and stored at ambient temperature and humidity until use.

Naegeli Dextrin

Naegeli dextrin from normal maize starch was prepared according to the method reported by Jane et al (1997). Starch was suspended in a 15% (v/v) sulfuric acid solution, held at 38°C for 12 days, and gently agitated each day during treatment. Upon completion of the hydrolysis period, Naegeli dextrin was washed with deionized water until all sulfuric acid was removed. Before use in this experiment, dried Naegeli dextrin was suspended in deionized water, collected by filtration on a Whatman No. 5 filter paper, and washed three times with 100% ethanol. The fluffy white powder was dried for 24 hrs at 32°C, weighed into 4 separate samples (0.700, 0.525, 0.350, and 0.175 g), and equilibrated at 100% relative humidity before X-ray analysis. After X-ray analysis, samples were stored in sealed vials until DSC analysis.

X-Ray Diffraction

Immediately before X-ray diffraction analysis, Naegeli dextrin and amorphous waxy maize starch samples were mixed in appropriate proportions for a total mass of 0.700 g. Mixtures were stirred and ground lightly with a mortar and pestle to make a homogenous powder. X-ray diffraction patterns were obtained using copper Kα radiation, graphite monochrometer filtered, with a Siemens D-500 diffractometer (Madison, WI, USA). Samples were scanned from 4 to 37 Two-Theta with a 0.05 Two-Theta step size and a 3 sec count time. Background determinations were made after smoothing with an 11-point parabolic filter. Areas were calculated using Shadow (Materials Data, Inc., Livermore, CA, USA) software. Percent crystallinity was calculated as the percent of area in the peaks relative to the total area.

Differential Scanning Calorimetry

Thermal properties were determined by using differential scanning calorimetry (DSC) equipped with an Intracooler II system and Pyris thermal analysis software (DSC-7, Perkin-Elmer, Norwalk, CT, USA). Sample (5 mg) and distilled water (40 or 50 μL) were combined in a ratio of 1:8 or 1:10 (starch:water), sealed in a stainless pan, and equilibrated for at least 1 hr. Samples were scanned from 25 to 150°C at a heating rate of 10°C/min using an empty pan as a reference with indium and cyclohexane as standards. Enthalpy change (ΔH), onset gelatinization temperatures (T_o), peak temperatures (T_p), and conclusion temperatures (T_c) were calculated automatically.

Results and Discussion

X-ray diffraction patterns of amorphous waxy maize starch, normal maize starch Naegeli dextrin, and mixtures thereof are shown in Figure 1. These diffractograms demonstrate that the

treated waxy maize starch was completely amorphous and the Naegeli dextrin was highly crystalline. Peak intensities increased, but the diffraction intensity at 12.5 Two-Theta from the amorphous background decreased as the percentage of Naegeli dextrin increased. Diffraction intensities for all samples were measured at 12.5 Two-Theta, and the relationship between diffraction intensity and Naegeli dextrin content was determined as shown in Figure 2. Diffraction intensities decreased linearly as the amount of Naegeli dextrin increased (R^2 = 0.9953). While degree crystallinity (percent crystalline material in the sample) appears to be related to diffraction intensity at 12.5 Two-Theta, it can only be calculated from diffraction intensity at 12.5 Two-Theta if a standard curve is prepared from samples of known degree crystallinity.

Diffraction intensity was measured at 12.5 Two-Theta because there were no nearby peaks in the diffractograms to interfere with the background, and it was early in the analysis so the samples remained relatively moist. Naegeli dextrin samples were equilibrated at 100% relative humidity (RH) before analysis, but amorphous samples were not. High humidity treatment of the amorphous starch may have provided opportunity for annealing or made the samples moist and sticky, thus causing technical difficulties in loading samples in the X-ray sample holder. X-ray diffraction was performed at ambient humidity (~50%), so Naegeli dextrin samples lost moisture during the course of X-ray diffraction analysis but amorphous portions of the mixtures presumably absorbed moisture.

Buléon et al (1987) reported that background and peak intensities differ with moisture content of starch samples. The moisture effect was examined by a simple experiment performed with potato starch. Potato starch was equilibrated at 52% RH or 100% RH before X-ray

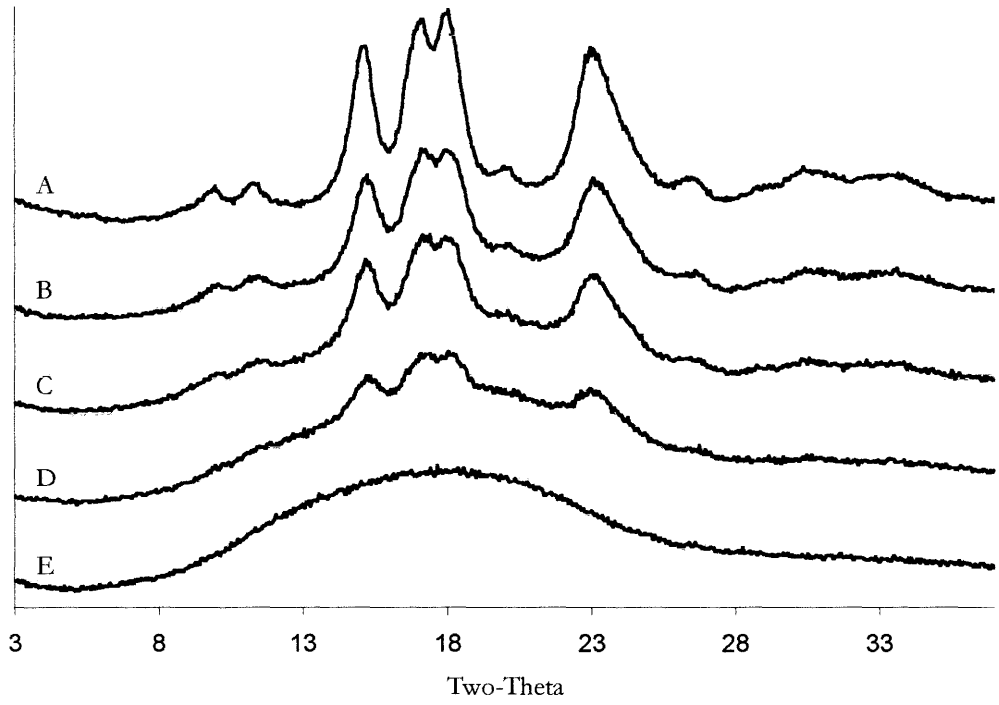

Figure 1. X-ray diffraction patterns for amorphous waxy maize starch-Naegeli dextrin mixtures. A= 100% Naegeli Dextrin, B= 25% amorphous, 75% Naegeli Dextrin, C= 50% amorphous, 50% Naegeli Dextrin, D=75% amorphous, 25% Naegeli Dextrin, E= 100% amorphous.

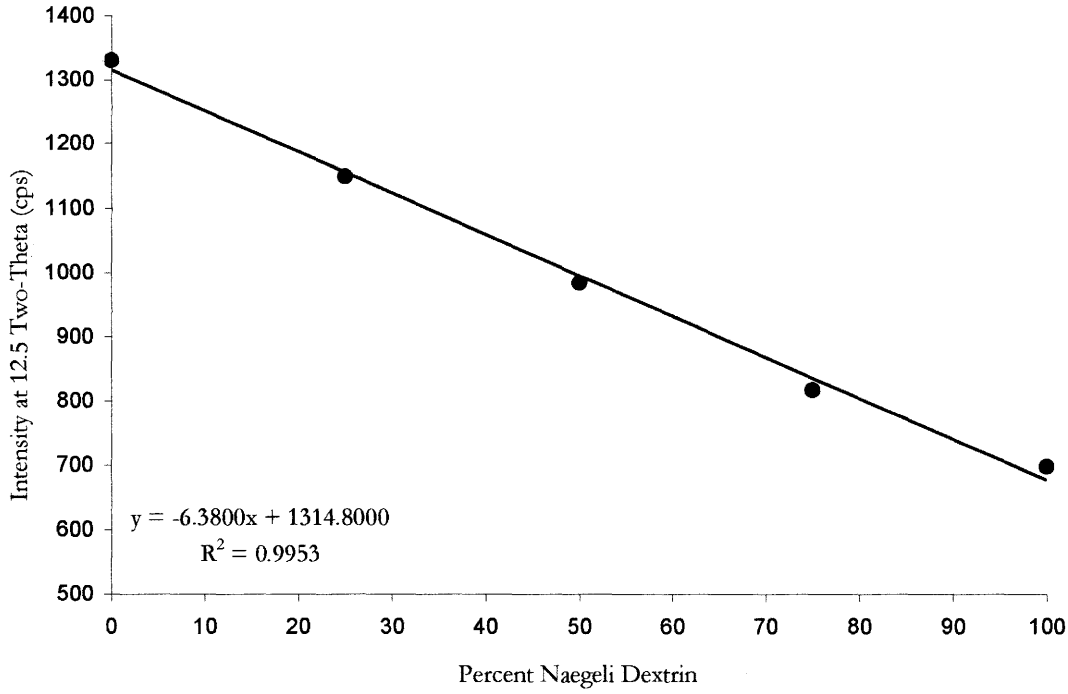

Figure 2. Naegeli dextrin content and background intensity at 12.5 Two-Theta.

analysis, and the degree of crystallinity was calculated to be 28 or 52%, respectively (degree of crystallinity was calculated by the area method that will be discussed later). Given the great impact of humidity treatment on calculated crystallinity, diffraction intensity at 12.5 Two Theta, early in the analysis, was used to avoid complication by any drying effect that may have been present in the samples.

Future experiments with this starch model system could be designed to avoid such a large drying effect. For example, both Naegeli dextrin and amorphous starch samples could be stored at ambient humidity, mixed together, placed in the X-ray sample holder, then equilibrated in 100% RH for 12-24 hours before analysis. X-ray analysis could be completed in a humidity-controlled environment at 100% RH. This method would avoid technical complications of loading saturated samples, and no drying would take place during X-ray analysis.

Peak area has long been used to calculate degree of crystallinity (Hermans and Weidinger 1948, 1961, Wakelin et al 1959). In this experiment, degree of crystallinity was calculated as the percent peak area of the total area. Results of this calculation for all samples are given in Table 1. The calculated degree crystallinity for Naegeli dextrin was 83.6%, which agreed well with the reported degree of crystallinity for potato Naegeli dextrin (80.3%) (Nara et al 1983).

Table 1. Degree crystallinity of Naegeli dextrin-amorphous waxy maize samples determined by peak area ratio to total area

Sample Composition		
Naegeli Dextrin (%)	**Amorphous (%)**	**% Crystallinity**
0.0	100.0	0.0
25.0	75.0	17.5
50.0	50.0	37.2
75.0	25.0	49.6
100.0	0.0	83.6

Figure 3 shows the relationship between sample Naegeli dextrin content and the calculated degree crystallinity. The sample mixtures containing 25 to 75% Naegeli dextrin demonstrated a linear relationship between proportion of Naegeli dextrin and degree of crystallinity with $R^2 = 0.99$, while all samples demonstrated a linear relationship with $R^2 = 0.97$. The observed degree of crystallinity for the 100% Naegeli dextrin sample (83.6%) was larger than would be expected from extrapolation of the line fitted to the other four samples (68.2%). The difference between the theoretical and observed degree of crystallinity in this sample may have been caused by the drying effect. Samples containing relatively dry amorphous starch and relatively moist Naegeli dextrin could exchange moisture between the components of the mixture, which may have led to accelerated drying of the Naegeli dextrin portion. The dried sample may have depressed the intensity of X-ray diffraction, thus leading to low crystallinity measurements.

Differential scanning calorimetry analysis was completed for all samples under two conditions: with starch to water ratios of 1:8 and 1:10. Amorphous starch was extremely sticky and absorbed much water, so 8 times mass of water may not have adequately hydrated all of the Naegeli dextrin in the mixed samples. The addition of 10 times of water appeared to adequately hydrate all samples. Endotherms from 1:8 and 1:10 starch:water ratios are shown in Figure 4 and Figure 5, respectively. Results of DSC analysis under both conditions are shown in Tables 2 and 3 for 1:8 and 1:10 starch:water ratios, respectively. Enthalpy change of melting clearly increased as the proportion of Naegeli dextrin increased, but onset temperature of melting was not well correlated to proportion of Naegeli dextrin.

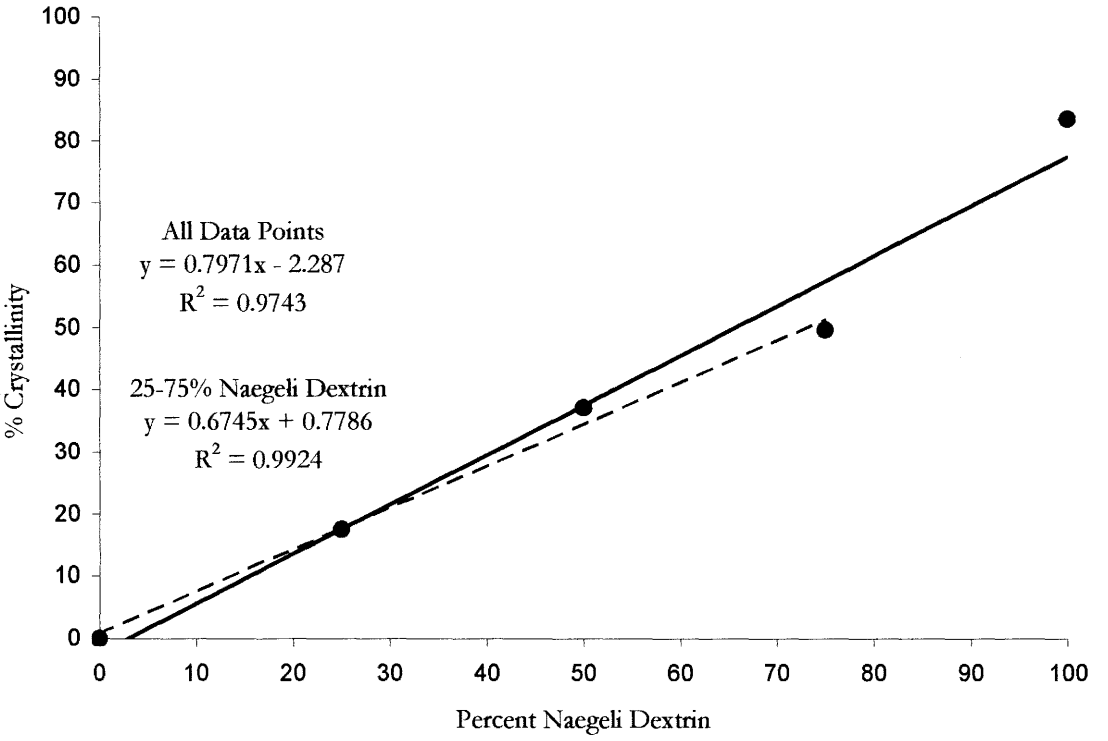

Figure 3. Crystallinity of amorphous waxy maize starch and Naegeli dextrin mixtures calculated as ratio of peak area/total area*100.

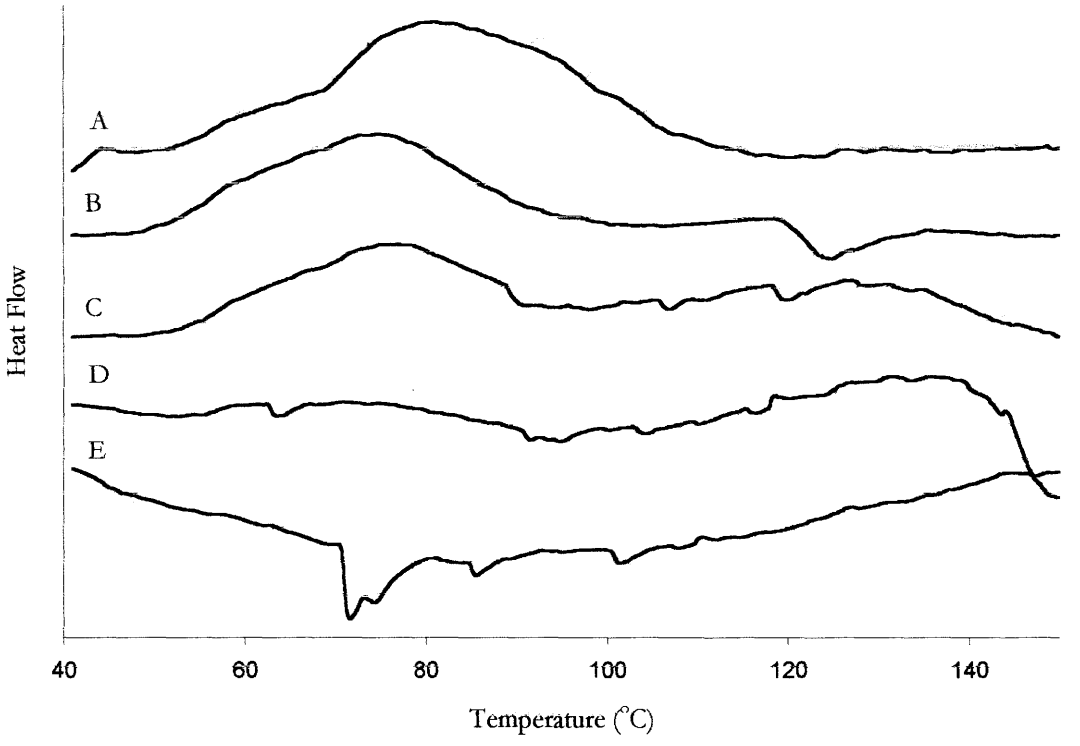

Figure 4. Endotherms of amorphous waxy maize starch and Naegeli dextrin (1:8 starch:water). A= 100% Naegeli Dextrin, B= 25% amorphous, 75% Naegeli Dextrin, C= 50% amorphous, 50% Naegeli Dextrin, D=75% amorphous, 25% Naegeli Dextrin, E= 100% amorphous.

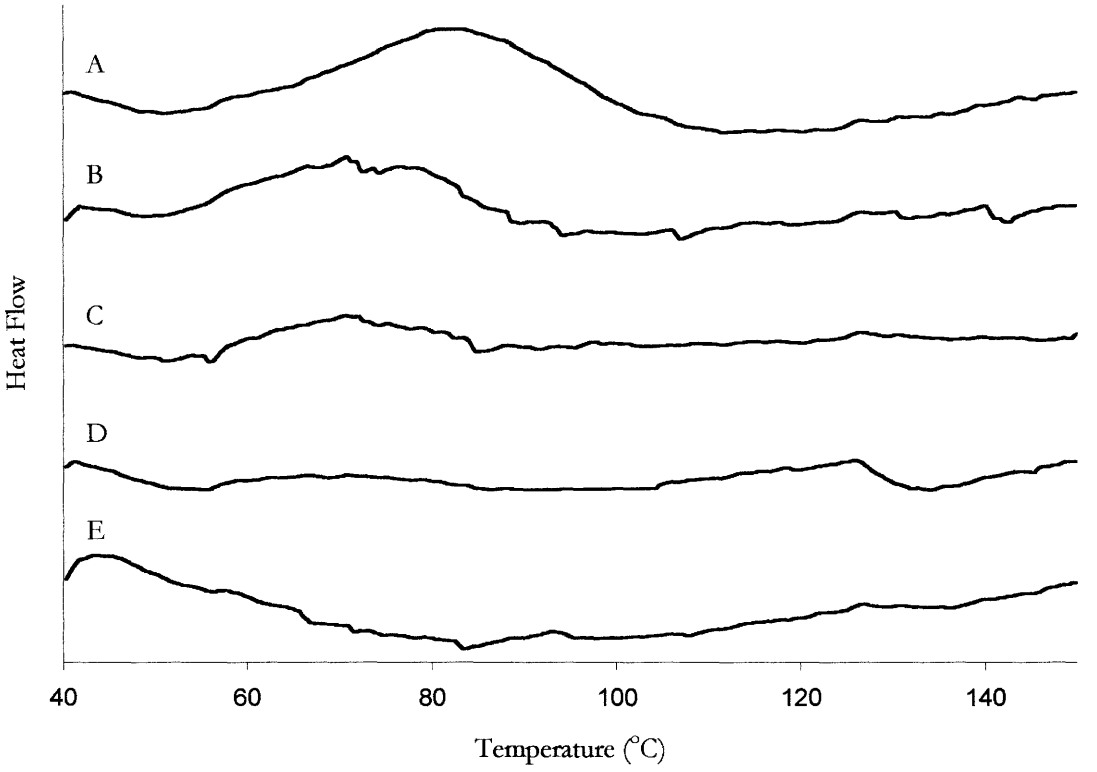

Figure 5. Endotherms of amorphous waxy maize starch and Naegeli dextrin (1:10 starch:water). A= 100% Naegeli Dextrin, B= 25% amorphous, 75% Naegeli Dextrin, C= 50% amorphous, 50% Naegeli Dextrin, D=75% amorphous, 25% Naegeli Dextrin, E= 100% amorphous.

Table 2. Thermal properties of Naegeli dextrin-amorphous waxy maize samples hydrated with water (1:8 starch:water)

Sample Composition					
Naegeli Dextrin (%)	**Amorphous (%)**	**T_o (°C)**	**T_p (°C)**	**T_c (°C)**	**ΔH (J/g)**
0.0	100.0	N/D	N/D	N/D	N/D
25.0	75.0	59.3 ± 5.9	75.0 ± 0.5	85.8 ± 7.6	2.3 ± 0.6
50.0	50.0	54.3 ± 0.6	75.0 ± 0.3	94.2 ± 1.1	7.3 ± 1.5
75.0	25.0	53.1 ± 1.6	74.7 ± 0.1	93.1 ± 0.1	12.0 ± 0.4
100.0	0.0	53.1 ± 0.7	81.7 ± 0.8	106.6 ± 3.0	18.5 ± 1.1

T_o T_p T_c = onset, peak, and conclusion temperatures of endotherm determined by differential scanning calorimetry. ΔH = enthalpy change of endotherm. N/D = Value not determined. Values are means of three determinations, mean ± standard deviation.

Table 3. Thermal properties of Naegeli dextrin-amorphous waxy maize samples hydrated with water (1:10 starch:water)

Sample Composition					
Naegeli Dextrin (%)	**Amorphous (%)**	**T_o (°C)**	**T_p (°C)**	**T_c (°C)**	**ΔH (J/g)**
0.0	100.0	N/D	N/D	N/D	N/D
25.0	75.0	55.8	70.7	84.0	1.6
50.0	50.0	55.5 ± 0.9	70.9 ± 05	83.2 ± 3.1	4.3 ± 0.7
75.0	25.0	53.7	75.2 ± 0.5	88.5 ± 1.4	7.4 ± 0.4
100.0	0.0	59.5 ± 1.7	83.3 ± 0.3	106.2 ± 2.5	13.4 ± 1.0

T_o T_p T_c = onset, peak, and conclusion temperatures of endotherm determined by differential scanning calorimetry. ΔH = enthalpy change of endotherm. N/D = Value not determined. Values are means of three determinations, mean ± standard deviation.

Figure 6 shows the relationship between percent Naegeli dextrin and enthalpy change of melting. The second order relationship between enthalpy change and percent Naegeli dextrin indicates the presence of an interaction between the two components of the mixed samples. Degree of crystallinity may be calculated from DSC enthalpy data (Wildmann and Reisen 1987), but enthalpy values for pure crystalline materials are needed. Such data were not available for this experiment.

Figure 7 demonstrates the relationship between peak melting temperature and sample composition and Figure 8 shows the relationship between onset melting temperature and sample composition. There was a second order relationship between the percent Naegeli dextrin and peak and onset melting temperatures, as would be expected for amorphous-crystalline polymer blends (Sperling 1992). Melting point depression of miscible polymer mixtures is a known phenomenon that has been successfully predicted with models of some polymer blends (Shenoy et al 1990). The DSC melting point and enthalpy of melting data collected in this experiment demonstrated that mixtures of crystalline and amorphous starch materials exhibited melting point depression. However, it is difficult to compare this starch model system to other well-studied polymeric model systems because this starch model system was composed of a solvent, and crystalline and amorphous materials; many other systems do not have solvents.

Conclusions

Two methods of analysis, DSC and X-ray diffraction, were used to measure crystallinity in a starch model system containing Naegeli dextrin as the crystalline portion and amorphous waxy maize starch as the amorphous portion. Both methods were shown to measure parameters related to degree of crystallinity. X-ray diffraction intensities at 12.5 Two-Theta linearly reflect

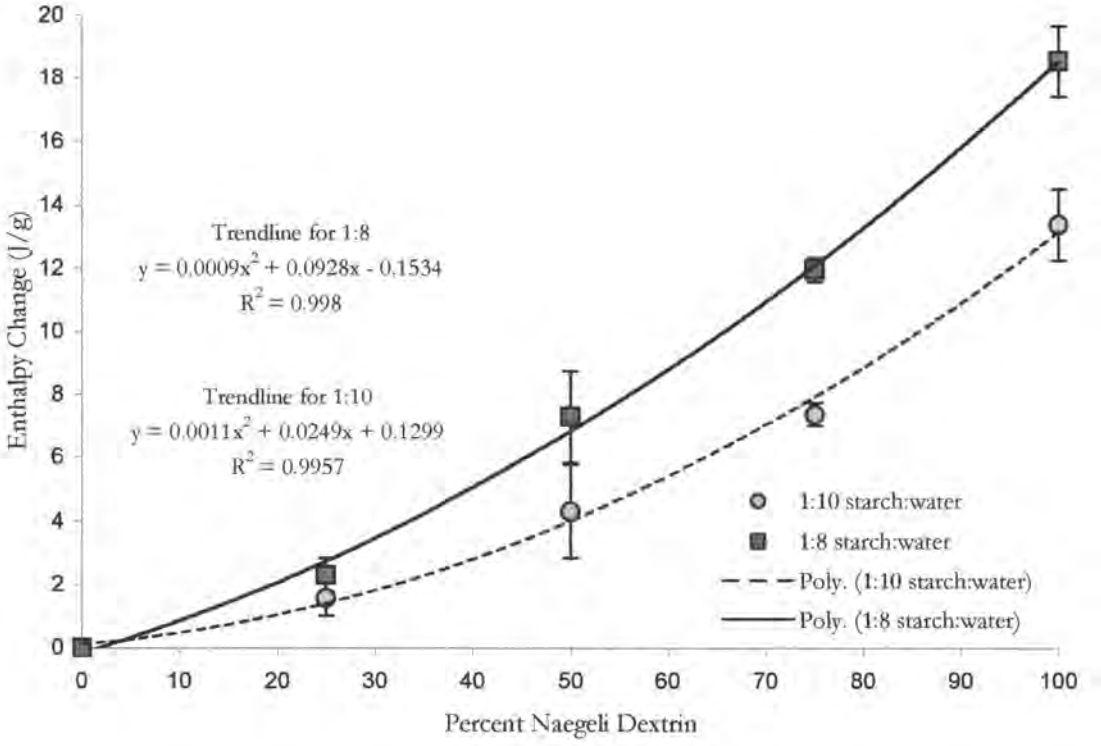

Figure 6. Enthalpy change in Naegeli Dextrin-amorphous starch mixtures determined by DSC.

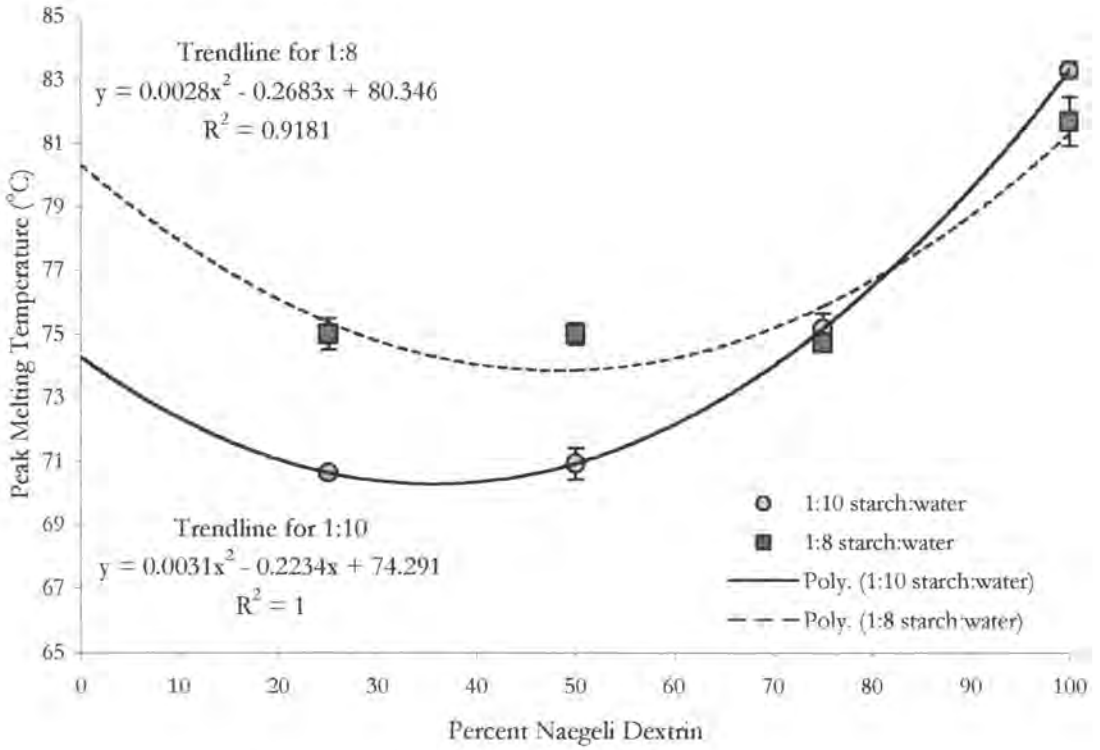

Figure 7. Peak melting temperature of Naegeli dextrin-amorphous starch mixtures determined by DSC.

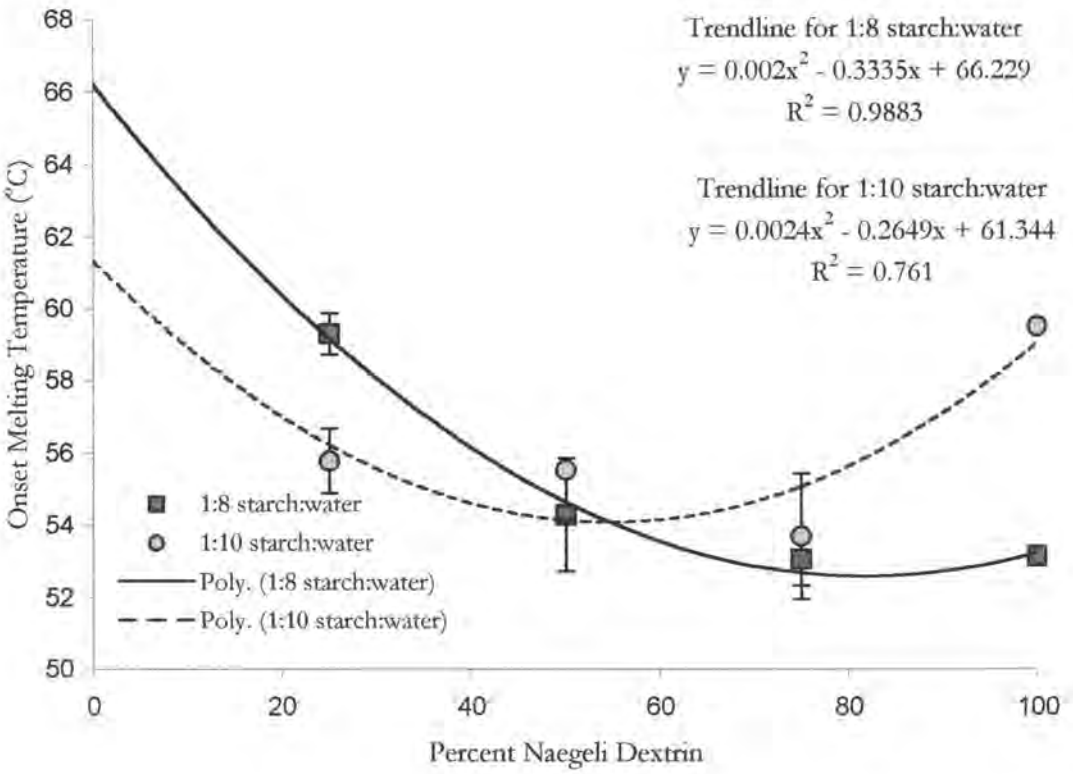

Figure 8. Onset melting temperature of Naegeli dextrin-amorphous starch mixtures determined by DSC.

Naegeli dextrin content of samples, and potentially could be correlated to degree of crystallinity. Peak areas could be used to calculate degree of crystallinity, but sample dehydration complicated the results. DSC analysis of samples in this model system showed that enthalpy change of gelatinization, onset and peak gelatinization temperatures were all affected by sample compositions in predictable ways. More precise information could be obtained by controlling the humidity of samples during X-ray diffraction analysis to prevent dehydration.

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EFFECTS OF GROWTH CONDITIONS ON AMOUNT AND STRUCTURE OF GLYCOGEN PRODUCED BY CYANOBACTERIA *SYNECHOSYSTIS PCC 6803*

Introduction

Glycogen is a glucose polymer that serves as a reserve energy source in mammals, bacteria, insects, fish, and other living organisms (Robyt 1998). The structure of glycogen is similar to that of starch amylopectin, the glucose storage substance found in plants, in that both molecules are made of α-1-4 linked glucose chains with α-1-6 linked branches. However, there are notable difference between the structures of glycogen and amylopectin. Glycogen is considered to be cold water-soluble and is highly branched (10%) while amylopectin in starch granules is insoluble in cold water and less branched (5%). The average branch chain length of glycogen is between 10 and 12 glucose units, and the average branch chain length of amylopectin between 19 and 31 (Jane et al 1999). The high degree of branching found in glycogen is key to the physiological function of glycogen. Glucose is released quickly and in large amounts when necessary, making glycogen an effective source of reserve energy in living organisms (Voet and Voet 1995).

Cyanobacteria are prokaryotes also known as blue-green algae that have been found to produce glycogen (Fogg 1956, Shively 1988). Glycogen is accumulated as storage material in the stationary stage of growth if the growth media contains an excess amount of carbon and a limiting amount of nitrogen, sulfate, or phosphate (Preiss 1969). This study was performed to determine how severely carbon and nitrogen limitation effect glycogen accumulation in cyanobacteria (*Synechocystis* PCC 6803). Cyanobacteria were grown in BG-11 media (Rippka et al 1979) with various amounts of glucose as the carbon source and sodium nitrate as the nitrogen

source. The amount of glycogen accumulated under four different conditions was measured by chemical and enzymatic methods. The molecular structure of glycogen extracted from bacteria grown under each media condition was examined by debranching with isoamylase and quantitative analysis of the branch chain length distribution.

Materials and Methods

Media and Growth Conditions

BG-11 liquid media was prepared according to the method reported by Rippka et al (1979). The media were prepared in three separate steps. First, a concentrated trace metal mix was prepared from the contents listed in Table 1. The trace metal mix was autoclaved for 20 minutes for sterilization, which resulted in the formation of a purple-colored precipitate.

Table 1. Trace metal mix for BG-11 cyanobacterial growth media

Chemical	Chemical Name	Grams/Liter	Molarity (mM)
H_3BO_3	Boric Acid	2.86	46.30
$MnCl_2 \bullet 4H_2O$	Manganese chloride	1.80	4.15
$ZnSO_4 \bullet 7H_2O$	Zinc sulfate	0.22	0.77
$Na_2MoO_4 \bullet 2H_2O$	Sodium molybdate dihydrate	0.39	1.61
$CuSO_4 \bullet 5H_2O$	Cupric sulfate	0.079	0.32
$Co(NO_3)_2 \bullet 6H_2O$	Cobalt (II) nitrate hexahydrate	0.0494	0.17

Second, a 100 times concentrated solution of BG-11 liquid media (100 X BG-11) was prepared and autoclaved for 20 minutes, or filter sterilized. The contents of this solution are listed in Table 2. Prepared 100 X BG-11 was clear to light purple in color. Ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA) buffer pH was adjusted to 8.0 with 50% (w/w)

sodium hydroxide. When nitrogen-limiting media was prepared, the amount of sodium nitrate was reduced from 149.58 grams (24.63 grams nitrogen per liter) to 8.38 grams for a final concentration of 1.38 grams of nitrogen per liter of 100 X BG-11 (Weber and Wober 1975). All other components remained unchanged.

Table 2. One hundred times BG-11 (100 X BG-11) cyanobacterial growth media

Chemical	Chemical Name	Quantity/Liter	Substitute
$NaNO_3$	Sodium nitrate	149.58[1]	94.1 g NH_4Cl
$MgSO_4 \bullet 7H_2O$	Manganese chloride	7.49 g	3.7 g anhydrous $MgSO_4$
$CaCl_2 \bullet 2H_2O$	Calcium chloride	3.60 g	2.72 g anhydrous $CaCl_2$
$C_3H_4OH(COOH)_3$	Citric Acid	0.60	
Na_2EDTA[2]	EDTA disodium salt dihydrate	1.12 mL of 0.25 M pH 8.0	
Trace Metal Mix		100 mL	

[1]Use 8.38 grams for nitrogen limiting media
[2]Ethylenediamine tetraacetic acid disodium salt dihydrate: $(HO_2CCH_2)_2NCH_2CH_2N(CH_2CO_2Na)_2 \bullet 2H_2O$

Table 3. BG-11 cyanobacterial growth media

Solution	Chemical Name	Stock Concentration	mL/L of BG-11
100 X BG-11		100 X	10
Tricine[1] Buffer pH 8.0	Tricine	1.0 M	5
Na_2CO_3	Sodium carbonate	50 mg/mL (189 mM)	1
K_2HPO_4	Potassium phosphate dibasic	30 mg/mL (175 mM)	1
	Ferric Ammonium Citrate[2]	6.0 mg/mL	1

[1]Tricine: $(HOCH_2)_3CNHCH_2CO_2H$
[2]Iron (III) ammonium citrate, structure undetermined, U.S. patent 2,644828, 1953

Third, BG-11 media were prepared from the 100 X BG-11 solution and the other ingredients listed in Table 3. Ferric ammonium citrate, sodium carbonate, and potassium phosphate stock solutions were filter sterilized rather than autoclaved for preservation. All

contents except the heat-sensitive ferric ammonium citrate solution were added before autoclaving. Ferric ammonium citrate was added after the media cooled to room temperature. A small amount of precipitate formed during autoclaving but the precipitate did not negatively influence the growth of cyanobacteria. Tricine buffer pH was adjusted to 8.0 with 50% (w/w) sodium hydroxide.

Supplements such as glucose and antibiotics were added to the BG-11 media according to the information listed in Table 4. These chemicals were added to autoclaved, cooled media because of heat sensitivity. Stock solutions of supplements were filter sterilized for preservation.

Table 4. Supplements to liquid or solid BG-11 cyanobacterial growth media

Chemical	Stock Solution	Final Concentration	mL/L of BG-11
Glucose	2.5 M (500 X)	5 mM	2
Tetracyclin	10 mg/mL	7.5 μg/mL	0.75
DCMU[1]	10 mM	10μM	1
Kanamycin	20 mg/mL	5-50 μg/mL	0.25-2.5
Spectinomycin	20 mg/mL	20 μg/mL	1
Sreptomycin	10 mg/mL	10 μg/mL	1
Chloramphenicol	15 mg/mL ethanol	7.5 μg/mL	0.5

[1]3-(3,4-dichlorophenyl)-N-N'-dimethylurea (DCMU)

Solid BG-11 media were prepared according to Table 5. The amounts listed in this table produced a total of 600 mL of solid media. Ferric ammonium citrate, glucose and/or antibiotics were added to the autoclaved media after it had cooled but before it solidified.

The liquid BG-11 media were prepared in, and the *Synechocystesis* sp. PCC 6903 cyanobacteria was grown in, Erlenmeyer flasks that were two to three times larger than the volume of media would have required. For example, one liter of media was prepared in a two-liter Erlenmeyer flask. Large flask sizes were required to prevent media loss during agitation on a

Table 5. Solid BG-11 cyanobacterial growth media

Solution	Chemical Name	Stock Concentration	mL/600 mL BG-11
100 X BG-11		100 X	6
Tricine[1] Buffer pH 8.0	Tricine	1.0 M	6
Na_2CO_3	Sodium carbonate	50 mg/mL (189 mM)	0.6
K_2HPO_4	Potassium phosphate dibasic	30 mg/mL (175 mM)	0.6
Agar			9.0 g (1.5%)
Add enough water to make 588 mL of media, autoclave			
	Ferric Ammonium Citrate[2]	6.0 mg/mL	0.6
$C_6O_6H_{12}$	Glucose[3]	2.5 M	1.2
$Na_2S_2O_3 \bullet 6H_2O$	Sodium thiosulfate	0.18g/ml (2.27 M)	10 mL

[1]Tricine: $(HOCH_2)_3CNHCH_2CO_2H$
[2]Iron (III) ammonium citrate, structure undetermined, U.S. patent 2,644828, 1953. Do not autoclave.
[3]Glucose addition is optional. Do not autoclave.

shaking surface, and it also allowed air and light to contact the bacteria. Two layers of aluminum foil were used to cap the flasks during autoclaving. The aluminum foil cover was loosened after inoculation to ensure that air could enter. Cyanobacteria were grown under constant fluorescent lighting on a shaking surface rotating at 125 rpm.

Media Comparisons

Wild type *Synechocystis* PCC 6803 were grown in four different media conditions with or without glucose (G+ or G-), and with sufficient or deficient amounts of nitrogen (N+ or N-) to investigate the relationship between glycogen production and the amount of glucose and nitrogen (in the form of sodium nitrate) in the growth media. A 50 mL glucose-containing, nitrogen-rich (G+ N+) pre-culture was inoculated with cells from a solid media plate and incubated for seven days. Five-milliliter aliquots of stock culture were transferred to four one-

liter flasks of G+ N+ media and incubated for seven days. The cells were collected by centrifugation (Sorvall RC-5C, Kendro Laboratory Products, Newtown, CT, USA) at 11,800 *g* for 15 min, rinsed three times with sterile deionized water (using centrifugation to collect cells after each rinse), and transferred to new media. The cells from each liter of G+ N+ media were transferred to one liter of new media, G+ N+, G+ N-, G- N+, or G- N-, and incubated in these different media for three days, then collected by centrifugation and stored at -18°C until analysis.

Growth Curves

Cyanobacteria *Synechocystis* PCC 6803 growth curves were measured for all four types of media. A stock culture was prepared by inoculating a 50 mL aliquot of G+ N+ media with several colonies of cells grown on solid media. The stock culture was grown for five days. Two hundred and fifty milliliters of new media (all four types) was inoculated with 0.5 mL of this stock culture and grown for 11 days. The optical density of the inoculated media was monitored daily by measuring the absorbance at 730 nm (Beckman DU 520, Fullerton, CA). Water was used as a blank.

Cell Breakage

Cyanobacteria *Synechocystis* PCC 6803 cells were broken by grinding under liquid nitrogen. Cells were collected by centrifugation (11,800 *g*, 15 min) and stored at -18°C over night to freeze the pellet and reduce the loss of cells during removal from the centrifuge tube. The pellet was ground under liquid nitrogen for 15 min or until the cells were a fine powder. Ten milliliters of water was added to the broken cells and the suspension was centrifuged (11,800 *g*, 30 min) to separate the water-insoluble cellular waste from the water-soluble cellular material. The supernatant was boiled for 10 min to inactivate enzymes (Bartley and Dean 1968), and the cooled supernatant was divided into two 5 mL aliquots so that glycogen could be harvested by

trichloroacetic acid treatment followed by ethanol precipitation or by ethanol precipitation alone.

Glycogen Extraction

Glycogen was extracted from broken bacterial cells following a method similar to the method reported by Weber and Wober (1975) using trichloroacetic acid (TCA) to precipitate proteins followed by ethanol precipitation of glycogen, or by ethanol precipitation only. Ethanol-only glycogen extraction was used to prevent any acid degradation of the glycogen that may have occurred during TCA treatment (Orrell and Bueding 1964). Five volumes of 100% ethanol was added to boiled and cooled supernatant from the broken cells. This solution was stored at 4°C over night and the blue precipitate was collected by centrifugation (9,000 *g*, 20 min) and air-dried at room temperature. Trichloroacetic acid treatment was performed following Weber and Wober's example. The boiled supernatant collected after centrifugation of broken cells was cooled and cold 20% TCA was added to a final concentration of 5%. Precipitated proteins were collected by centrifugation (9,000 *g*, 20 min). Five volumes of 100% ethanol was added to the remaining supernatant and this solution was stored at 4°C over night, during which time a fluffy white precipitate formed. The TCA-soluble glycogen precipitate was collected by centrifugation (9,000 *g*, 20 min) and allowed to air-dry at room temperature. The TCA-insoluble fraction that precipitated upon TCA addition was treated with 5 mL of 0.1 M sodium hydroxide and mixed until all of the precipitate dissolved (Fujimori et al 1995). Five volumes of 100% ethanol was added and the solution was stored at 4°C over night. The blue precipitate was collected by centrifugation (9,000 *g*, 20 min) and dried at room temperature.

Glycogen Content Determination

The precipitate that was collected and dried after ethanol-only treatment was dissolved in 1.0 mL of deionized water. The TCA-insoluble fraction, which precipitated after ethanol

addition, was dissolved in 1.0 mL of deionized water. The dried TCA-soluble glycogen pellet was dissolved in 0.5 mL of deionized water. The glycogen content of these three solutions was analyzed chemically and enzymatically. Total carbohydrate analysis by the phenol-sulfuric acid method was used to determine the amount of carbohydrate present (Dubois et al 1956). The amount of glycogen present was determined by an amyloglucosidase enzymatic method. One-hundred microliters sample, 100 μL of 0.1 M acetic acid buffer, pH 4.5, and 5 μL of amyloglucosidase solution (133 units/mL) were combined and incubated for 2 hours in a 55°C shaking water bath, 150 rpm. A Sigma Glucose Analysis Kit was used to determine the amount of glucose released from the enzymatic degradation of glycogen.

Protein Content Determination

Protein content of the precipitated glycogen was determined by combining 50 μL of sample with 2.50 mL of Bradford reagent (Bradford 1976). Colorimetric measurements were taken at 595 nm (Beckman DU 520, Fullerton, CA).

Branch Chain Length Distribution Determination

Cyanobacterial glycogen was debranched with isoamylase following previously published methods, with slight modifications (Jane and Chen 1992). A small scale debranching procedure was followed for the cyanobacterial glycogen because of the limited amount of glycogen sample available. The total volume of the isoamylase solution was 200 μL and the pH was adjusted to 6-7 with 0.1 N sodium hydroxide added in 3.0 μL aliquots. The pH was monitored by using the tip of a 20 μL capillary-action pipette to take a small sample, which was expelled onto a piece of pH paper for pH measurement. After neutralization and boiling, the samples were filtered through microcentrifuge tube cellulose acetate filters (Corning Inc., Corning, NY). Branch chain length distributions were analyzed by using a high-performance anion exchange chromatography

system equipped with an enzyme column reactor and a pulsed amperometric detector (Dionex, Sunnyvale, CA) (HPAEC-ENZ-PAD) by the method reported by Wong and Jane (1997).

Results and Discussion

Growth curves of *Synechocystis* PCC 6803 grown in the four types of media types used in this study are shown in Figure 1. This figure demonstrates that G+ N+ media produced the greatest absorbance at 730 nm in the stationary phase and G- N- produced the least. The stationary phase of *Synechocystis* PCC 6803 in G+ N+ media had an absorbance at 730 nm of approximately 1.7, which was more than twice that of the other three types of media. While the absorbance measurement did not directly quantify the cellular concentrations, it did indicate that the glucose-containing, nitrogen-rich media promoted the most abundant cellular growth.

Four one-liter flasks of G+ N+ media were inoculated with *Synechocystis* PCC 6803 and allowed to grow for seven days. The cells were isolated and transferred to four different types of media as described previously. The average growth curve for the four flasks of G+ N+ before transfer is shown in Figure 2 and the individual growth curves after transfer are shown in Figure 3. The growth curves in Figure 3 demonstrate that the glucose-containing media produced increased absorbance at 730 nm immediately after transfer, followed by a slight decrease. On the contrary, glucose-deficient growth conditions demonstrated a slight decrease in absorbance directly after transfer followed by a slight increase.

The cells were collected, broken, and treated with trichloroacetic acid (TCA) or ethanol only to produce three fractions: TCA-soluble, TCA-insoluble, or ethanol-precipitated as described above. The carbohydrate (glycogen and any other water-soluble carbohydrates)

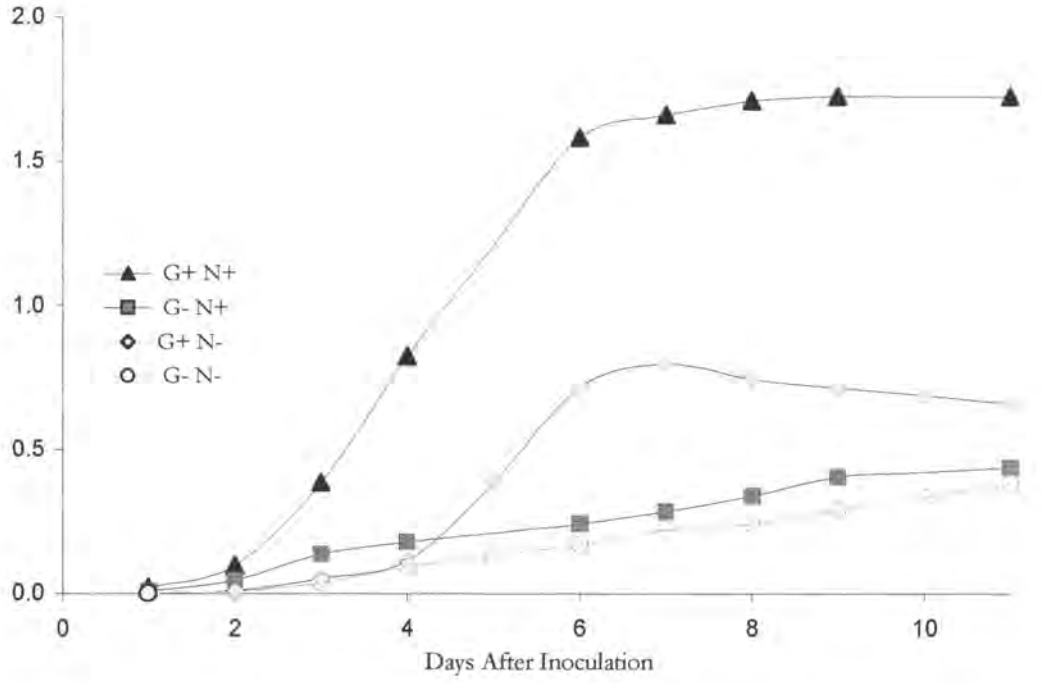

Figure 1. Growth curves of cyanobacteria in various media conditions

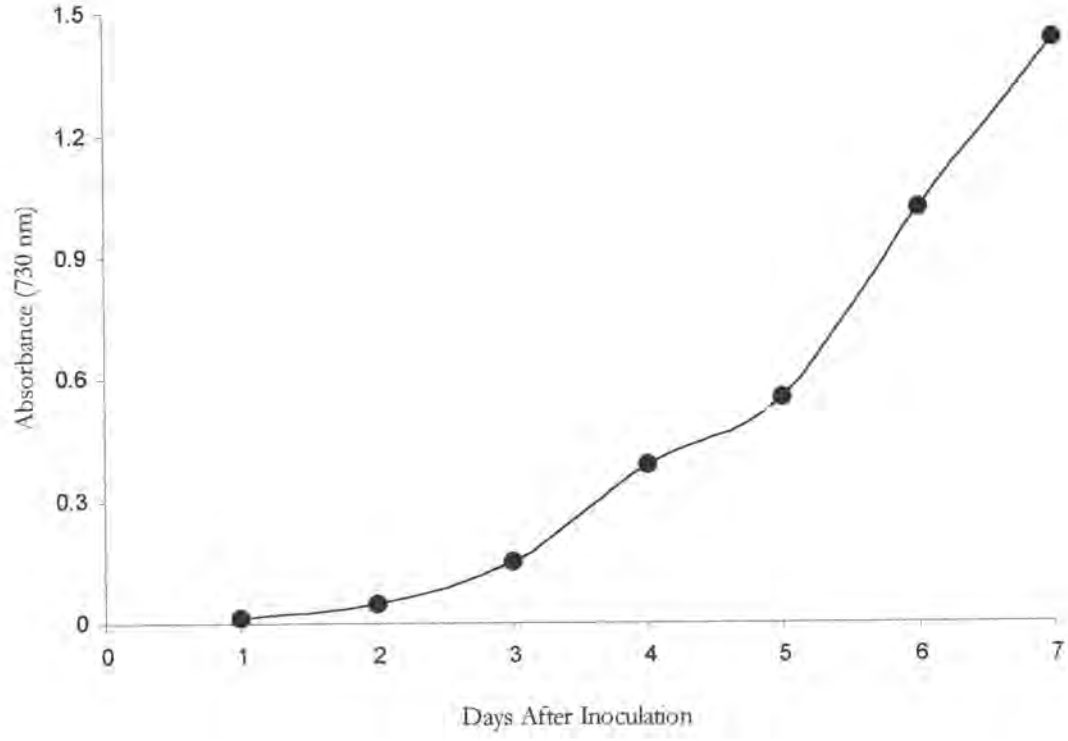

Figure 2. Average growth curve of cyanobacteria in G + N + media

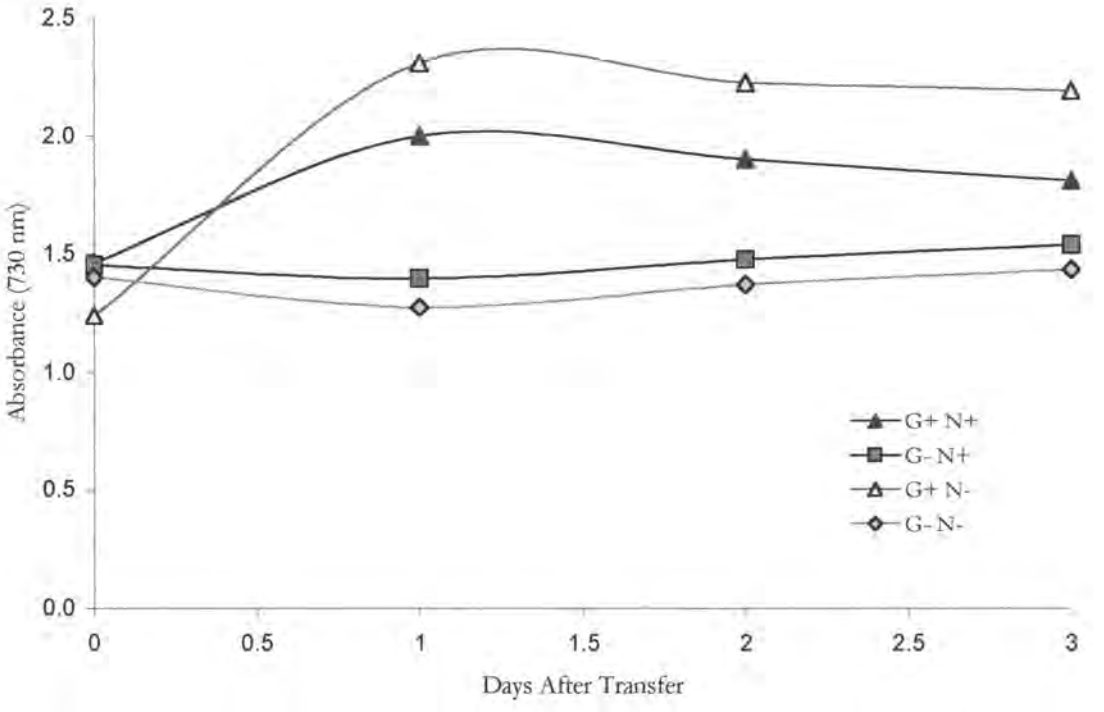

Figure 3. Growth curves of cyanobacteria after transfer to various media conditions

extracted from one liter of media was quantified by the phenol-sulfuric acid method and the results are shown in Table 6. The glucose-rich, nitrogen-deficient (G+ N-) media produced the most carbohydrate, followed by G+ N+, G- N+, and G- N-. The amount of carbohydrate present in the TCA-soluble fraction was much larger than that present in the TCA-insoluble fraction for both of the glucose-containing media. However, there was more carbohydrate in the TCA-insoluble fraction than the TCA-soluble fraction for both of the glucose-deficient conditions.

Enzymatic determination of glycogen accumulated under each media growth condition was performed and the results of this analysis are listed in Table 7. The most glycogen was accumulated under the G+ N- media condition, followed by the G+ N+, G- N+, and G- N- media conditions. In comparison to Table 6, the amount of glycogen in each fraction determined enzymatically was generally smaller than the amount of carbohydrate determined chemically. The enzymatic quantification of glycogen in the TCA-soluble fraction from the G+ N- media condition was smaller than would be expected, which may have been caused by incomplete enzyme hydrolysis of the glucose in this fraction. However, the enzymatic method of glycogen determination was considerably variable. The sums of glycogen in the TCA fractions were 92.5, 42.4, 72.3, and 65.8% of the glycogen in the ethanol only fraction for G+ N+, G+ N-, G- N+, G- N- media conditions, respectively. Total carbohydrate quantification by the phenol-sulfuric acid method was also variable, and sums of carbohydrate in the TCA fractions were 80.2, 90.2, 67.6, and 105.5% of the carbohydrate in the ethanol only fraction for G+ N+, G+ N-, G- N+, G- N- media conditions, respectively.

The protein content of glycogen extracted from cyanobacteria grown under each media condition was determined using Bradford reagent and the outcomes of this study are shown in

Table 6. Total carbohydrate determination of glycogen yield from *Synechocystis* PCC 6803 grown under various media conditions

Extraction Method	G + N + (mg/L)	G + N - (mg/L)	G – N + (mg/L)	G – N - (mg/L)
Ethanol	18.07	29.98	2.10	1.09
TCA-Soluble	12.40	20.04	0.24	0.20
TCA-Insoluble	2.10	6.99	1.18	0.95

Table 7. Enzymatic determination of glycogen yield from *Synechocystis* PCC 6803 grown under various media conditions

Extraction Method	G + N + (mg/L)	G + N - (mg/L)	G – N + (mg/L)	G – N - (mg/L)
Ethanol	16.22	23.52	1.12	1.17
TCA-Soluble	11.76	11.92	0.29	0.07
TCA-Insoluble	3.24	1.94	0.52	0.70

Table 8. These results indicate that trichloroacetic acid was a successful method of removing proteins from glycogen. The amount of protein in the ethanol-only treated samples was comparable to the amount of protein in the TCA-insoluble fraction of the TCA-treated samples. There was no detectable protein in the TCA-soluble fraction.

Branch chain length distributions of *Synechocystis* PCC 6803 glycogen extracted by ethanol only treatment and TCA treatment were prepared by isoamylase treatment. The TCA-soluble glycogen was analyzed for all four media conditions, but the ethanol only treated glycogen was analyzed for only three media conditions, as insufficient glycogen was extracted from the G- N+ media. Figure 4 shows the branch chain length distributions of TCA-treated glycogen. There were distinct differences in the branch chain length distributions of glycogen extracted from bacteria grown in the various media. For example, glycogen from bacteria grown in G+ N- media had the longest long branches with degree of polymerization (DP) 48 while glycogen from bacteria grown in G- N+ media had the shortest long branches (DP 26). Both of the media that contained glucose produced glycogen with longer branch chains than did the glucose-deficient media, but the average branch chain length was larger for nitrogen-deficient media than for nitrogen-abundant conditions. The longest branch chain lengths, peak (most abundant), and average chain lengths for debranched TCA-treated glycogen samples are given in Table 9.

Figure 5 shows the branch chain length distributions of ethanol only-treated glycogen extracted from *Synechocystis* PCC 6803 grown under various media conditions. As with the TCA-treated glycogen samples, branch chain length distributions are distinctly different for glycogen extracted from bacteria grown under different media conditions. The glucose-rich media conditions resulted in the production of glycogen with longer branch chain lengths than did the glucose-deficient media. The longest detectable branch chain length was DP 46 for

Table 8. Protein content of glycogen from *Synechocystis* PCC 6803 grown under various media conditions

Extraction Method	G + N + (mg/L)	G + N - (mg/L)	G – N + (mg/L)	G – N - (mg/L)
Ethanol	2.08	2.60	2.38	1.56
TCA-Soluble	0.00	0.00	0.00	0.00
TCA-Insoluble	2.86	2.30	2.12	1.78

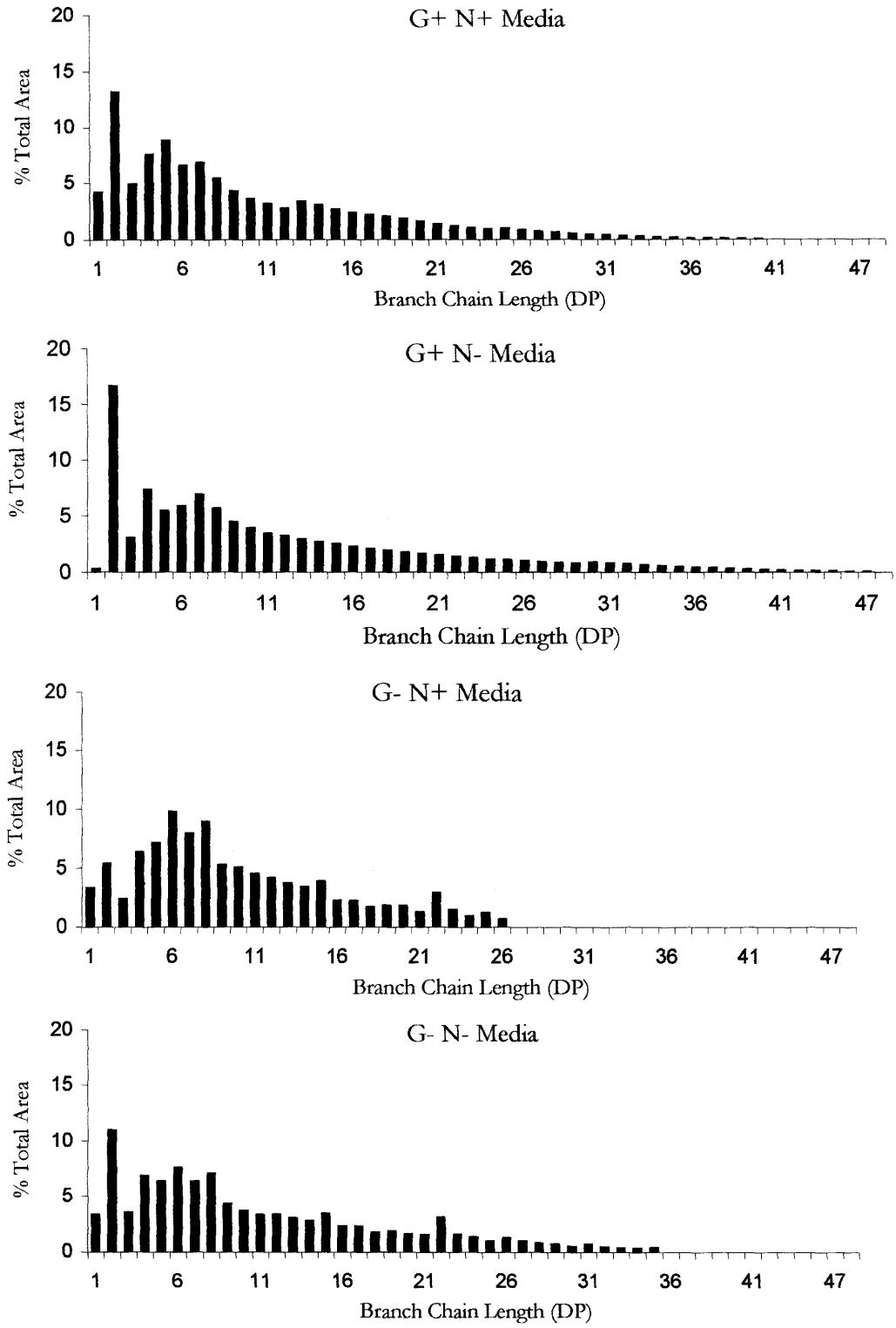

Figure 4. Branch chain length distributions of TCA-extracted glycogen extracted from cyanobacteria *Synechocystis* PCC 6803 grown under various media conditions.

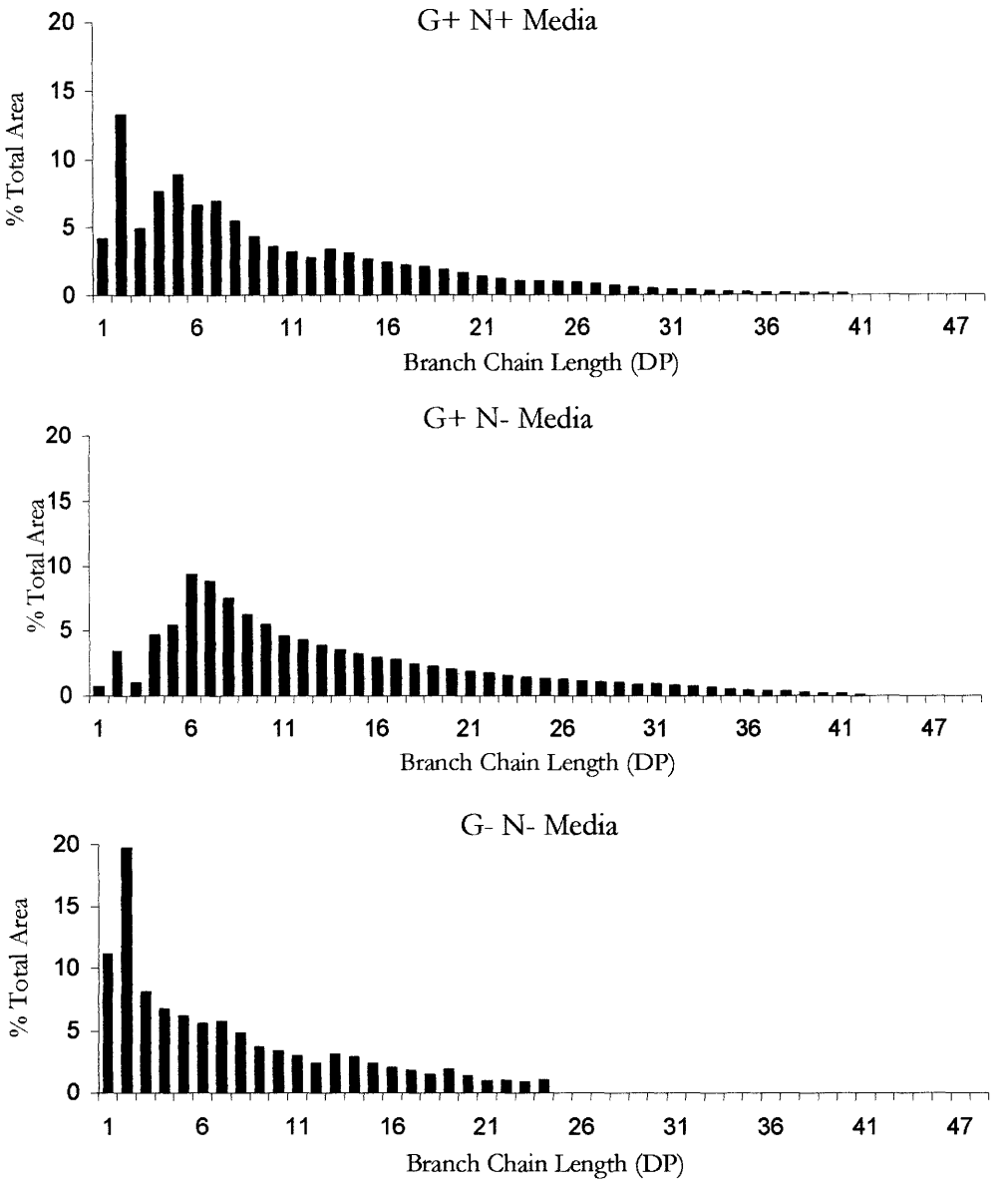

Figure 5. Branch chain length distributions of ethanol-precipitated glycogen extracted from cyanobacteria *Synechocystis* PCC 6803 grown under various media conditions.

Table 9. Branch chain length distributions for debranched TCA-treated glycogen extracted from *Synechocystis* PCC 6803 grown in various media conditions

Media Condition	Peak Branch Length (DP)	Longest Branch Length (DP)	Average Chain Length (DP)
G+ N+	2	43	9.53
G+ N-	2	48	11.24
G- N+	6	26	9.95
G- N-	2	35	10.71

glycogen from bacteria grown in G+ N+ media and DP 43 for glycogen extracted from bacteria grown in G+ N- media. Average branch chain lengths were larger for both glucose-rich media conditions than for the G-N- media condition. In the TCA-treated samples, it appeared as though average branch chain length was related to nitrogen deficiency rather than glucose content, but with ethanol-only treated samples, it appeared as though average branch chain length was related to glucose content. The peak, longest, and average chain lengths for glycogen extracted by ethanol only treatment are listed in Table 10. Note that data in the table do not always appear to match the figures because the instrument was able to detect very small amounts of long chains, and these small amounts may not be shown on the histograms.

Table 10. Branch chain length distributions for debranched ethanol only-treated glycogen extracted from *Synechocystis* PCC 6803 grown in various media conditions

Media Condition	Peak Branch Length (DP)	Longest Branch Length (DP)	Average Chain Length (DP)
G+ N+	2	46	10.41
G+ N-	6	43	12.67
G- N-	2	24	7.13

The branch chain length distribution profiles of cyanobacterial glycogen demonstrated that the structure of glycogen produced by *Synechocystis* PCC 6803 was altered by the media in which the bacteria were grown. Glucose-containing media resulted in the production of glycogen with longer branch chain lengths than did glucose-deficient media. This finding that was consistent for both TCA and ethanol-only treatment during glycogen extraction and purification.

Conclusions

Cyanobacterial growth and glycogen accumulation were greatly affected by the nutrient content of the media in which the bacteria were grown. Growth curves from *Synechocystis* PCC 6803 in various media demonstrated that these bacteria grew the fastest and caused the highest absorbance at 730 nm when grown in glucose and nitrogen-rich media, followed by glucose-rich, nitrogen-deficient media.

Studies of glycogen accumulation under different growth conditions demonstrated that the amount of glucose and nitrogen in the media influenced the amount of glycogen produced. The largest amount of glycogen was accumulated in glucose-rich, nitrogen-deficient media. Not only was the amount of glycogen influenced by the nutrient composition of the media in which the bacteria were grown, the branch structure of the glycogen also changed with the media composition. Glucose-rich media resulted in glycogen with longer branch chain lengths than glucose-deficient media, but for TCA-treated samples, glycogen from bacteria grown under nitrogen-deficient conditions had longer average chain lengths. The extraction and purification procedure appeared to influence the average branch chain length distribution of glycogen and the amount of protein present in the glycogen. Trichloroacetic acid treatment proved to be an

effective way to remove proteins from glycogen, producing more pure glycogen samples without major damage to the glycogen molecular structure.

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GENERAL CONCLUSIONS

Isolated maize and Chalco teosinte starches were virtually indistinguishable in some aspects. For example, Chalco teosinte and maize starches demonstrated similar X-ray diffraction patterns, degrees of crystallinity, amylopectin molecular weights, and amylose contents. However, the starches were obviously different in granular form, determined by scanning electron microscopy, and Chalco teosinte starch had shorter branch chains, lower gelatinization temperature, higher retrogradation rate, and lower peak viscosity followed by greater shear-thinning than maize starches. However, Chalco teosinte starch was more similar to maize starch than to other cereal starches. Uses of starch in commercial food applications often require low retrogradation (staling) rates and high peak viscosities. Maize starch demonstrates these qualities more than Chalco teosinte starch, potentially making it a more valuable starch, and the BSSS-maize-Chalco teosinte cross properties were generally similar to those of BSSS maize. If, in fact, maize descended from teosinte as Dr. George Beadle proposed, then natural selection followed by careful breeding has produced a plant with more desirable starch.

The semi-crystalline structure of starch granules accounts for many of the unique properties found in starch. Crystallinity and melting properties of starch were studied with the use of a starch model system and demonstrated that mixtures of amorphous and crystalline starch materials behaved in predictable ways. The degree of crystallinity increased linearly with Naegeli dextrin (83% crystallite) content when calculated as X-ray diffraction intensity at 12.5 Two-Theta or as a ratio of the peak area to total area. However, moisture content and drying caused difficulties in X-ray diffraction analysis. Differential scanning calorimetry analyses showed second order increases in enthalpy change of gelatinization and peak gelatinization

temperature with linear increases in Naegeli dextrin content. The second order relationships indicate the possibility of an interaction between the crystalline and amorphous components of the mixtures. A more controlled experimental set-up could eliminate the drying effect observed in the X-ray patterns, and thus give more accurate results. Also, studies using whole starch granules would further confirm the correlation between degree of crystallinity, melting temperature, and melting enthalpy in starches.

Glycogen, a non-crystalline, cold water soluble amylopectin-like glucose storage molecule produced by cyanobacteria *Synechocystis* PCC 6803 was produced most abundantly in glucose-rich, nitrogen-deficient media. However, the bacteria grew the fastest in glucose-rich, nitrogen-rich media. Glycogen branch chain length distributions changed with the media composition, and glucose-rich media (nitrogen-rich or deficient) resulted in glycogen with longer branch chain lengths than glucose-deficient media conditions. The glycogen purification procedure using trichloroacidic acid did not appear to influence on the branch chain length distribution of glycogen, but it did reduce the amount of protein present in the glycogen. Therefore, media conditions are important in determining the amount and structure of glycogen produced by cyanobacteria, but extraction procedures did not have a noticeable affect on these glycogen properties.

ACKNOWLEDGMENTS

I would like to thank Dr. Jay-lin Jane, my major professor, Dr. Pamela White, and Dr. John Robyt, my committee members, for their guidance and instruction through my graduate studies. I am very grateful to the USDA and the Iowa Corn Promotion Board for their financial support. Dr. Kit-sum Wong, thank you for all of your help in the lab. I could not have accomplished this with out you. Thanks to Sang-ho Yoo and David Stevenson for your help with research and graduate life, and a special thanks to Dr. Scott Schlorholtz for all of your encouragement and help with the X-ray work. I am grateful to Dr. Martin Spaulding and his group for the use of laboratory space for growing cyanobacteria, and to Ben Voss for helping so much with the bacteria work. Most importantly, thank you, Nicholas, for your love, support, and patience through this experience.