DISSERTATION

IMPORTANCE OF ZEIN COMPOSITION IN QUALITY PROTEIN MAIZE (QPM) TO MICROSTRUCTURAL AND CHEMICAL MODIFICATIONS OF PROTEINS DURING EXTRUSION AND THEIR IMPACT ON TEXTURE OF CORN-BASED EXTRUDATES

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

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Fall 2002

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COLORADO STATE UNIVERSITY

September 13, 2002

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY MARIA CRISTINA DIAS PAES ENTITLED IMPORTANCE OF ZEIN COMPOSITION IN QUALITY PROTEIN MAIZE (QPM) TO MICROSTRUCTURAL AND CHEMICAL MODIFICATIONS OF PROTEINS DURING EXTRUSION AND THEIR IMPACT ON TEXTURE OF CORN-BASED EXTRUDATES BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

IMPORTANCE OF ZEIN COMPOSITION IN QUALITY PROTEIN MAIZE (QPM) TO MICROSTRUCTURAL AND CHEMICAL MODIFICATIONS OF PROTEINS DURING EXTRUSION AND THEIR IMPACT ON TEXTURE OF CORN-BASED EXTRUDATES

Although several studies have been already carried out to clarify the importance of endosperm corn proteins in corn grain hardness and vitreousness, the role that protein compositional differences plays on the physical properties of texturized corn-based products is still poorly understood. Considering that protein has been shown to have an important impact in extruded food systems, it is important to understand how differences in composition or spatial arrangement of corn endosperm proteins, such as observed in Quality Protein Maize (QPM), can influence changes in polymers and structures during extrusion processing. Therefore, the overall objective of this project was to investigate the effect of the high γ-zein content in QPM to chemical, physical, and microstructural changes that occurs in zeins during extrusion and its impact on texture of intermediate corn-based extrudates. Three experiments were designed to test the overall scientific hypothesis that high γ-zein content in QPM influences chemical modifications of proteins

during extrusion and provides different textural properties in corn-based extrudates as compared to normal corn.

Overall results of thermal and pasting properties for starch isolated from QPM and normal maize grits showed either no or negligible differences between starch of normal and QPM maize types. This finding allowed maize grits to be used in prospective studies for comparison of possible modifications in proteins during processing.

Extrusion caused significant changes in the microstructural organization of major components of the grits and the chemistry of maize proteins. Starch granules lost their orderly structure, gelatinized, and melted to form a continuous matrix, whereas, proteins aggregated into large masses diffused in the gelatinized starchy network. The high γ-zein content in QPM seems to confer resistance to disruption of protein bodies during extrusion. Therefore, high γ-zein rich protein bodies require higher energy input within the extruder for complete disruption than protein bodies found in normal maize. But differences in dispersion of zeins in the system for the two maize types were not identified at high shear conditions evaluated in this study. In general, modifications in protein chemistry were driven by both noncovalent and covalent interactions, although disulfide bonding was apparently more important for the QPM samples.

Chemical changes in proteins were identified as an important factor influencing brittleness of the final extrudates. Moisture negatively affected the brittleness of extrudates, regardless of maize type. Higher content of γ-zein in QPM likely provided higher strength to maize extrudates thus favoring crispness of extruded maize grits. The

use of QPM grits to replace normal grits in the production of extruded corn-based products may promote extended bowl-life of extruded corn flakes as well as enhance crispness of fully expanded snacks.

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ACKNOWLEDGMENTS

My sincere acknowledgment to the Brazilian Agriculture Research System-EMBRAPA for having sponsored my training. I also would express my deepest gratitude to my committee members: Dr. Martha Stone, my advisor, for her words of encouragement and valuable guidance through my graduate program; Dr. Bruce Hamaker, my co-advisor and professor at Purdue University, for kindly sharing his knowledge and time with me, and providing financial support and guidance for this research project; Dr. Lucy Liangly Yu, and Dr. John Chandler for their suggestions for completion of this dissertation.

My special thanks to Dr. John Lawton at USDA/ARS/NCAUR in Peoria, IL, and his coworkers, who assisted me with the extrusion work. Also, thanks to Debra Sherman and Chia Ping Huang at the Electron Microscopy Center at Purdue University, and Dr. Guy Capdeville at Embrapa Genetic Resources, who helped me to accomplish the electron microscopy study.

To Dr. Miriam Terezinha Eira, Dr. Antonio Alvaro Purcino, Dr. Isabel Souza, and Dr. Regina Modesta, colleagues at Embrapa, thanks for their support and words of encouragement. Also, my appreciation goes to Heliete and Edna for providing me with their help during the lab work.

I acknowledge my colleagues at the Cereal Science laboratory at Purdue University for their words of encouragement. Thanks to Dr. Adam Aboubacar for his

valuable advice on procedures applied in this work. Also, my great gratitude to Laurie Scanlin, Tina Parker, and Ahuva Sadeh for their support and friendship.

My special thanks to my family members for their unfailing support, love, and prayers, especially to my parents who always instilled the best in me.

Most of all, my deepest thank you is to my husband, Decio Karam, for his love, understanding, dedication, and encouragement.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

CHAPTER I

LITERATURE REVIEW

Introduction

Corn, also known as maize, is the second major produced cereal grain in the world with the United States, China, and Brazil as the largest worldwide producers (FAO 2002). Total maize production in the United States in 2001 was approximately 241 million tons, while 41 million tons were harvested in Brazil in the same growing season (FAO 2002). In both Brazil and America corn consumption is primarily as feed for livestock. Industrial use differs in these countries since wet milling is the primary method for corn processing in North America (Corn Refiners Association 1999), while dry milling is the most important industrial corn process in Brazil (ABIMILHO 2002). It is estimated that 11% of the total corn consumed in Brazil will be industrially processed this current year (Safras e Mercados 2002). About 70% of this total will be dry milled, resulting in the production of corn grits and corn flours of various particle sizes (ABIMILHO 2002). These products are primarily destined for expanded snacks and ready-to-eat breakfast cereals through extrusion processing, a growing trend for the last 10 years in Brazil (ABIA 2001) and in the United States (Snack Food Association 2000). Despite the fact that corn is primarily processed through the wet milling method in the

United States, the American food industry manufactured 1.4 billion pounds of tortilla chips and 272.4 million pounds of corn chips in 1999 (Snack Food Association 2000).

In addition to extrusion variables, the starch fraction of the corn kernel is the major component responsible for the physical properties of cereal-based extruded products, such as corn flakes and corn-based savory snacks (Moore 1994; Riaz 1997). However, proteins, mainly the alcohol soluble fraction zein, have been recently shown to influence the structure and texture of corn-based extrudates (Batterman-Azcona 1998). In extruded cornflakes production, zeins are released from the constraint of the protein bodies and can interact with each other (Batterman-Azcona and Hamaker 1998). This may alter texture and bowl-life of cornflakes (Tandjung 2000), two important attributes that affect cornflake quality. Despite the importance of this recently reported information on processing of extruded and flaking corn products, it is still not known if a modified zein pattern, such as the high γ-zein reported for the specialty corn, Quality Protein Maize (QPM), could influence the chemical modifications of proteins during extrusion and affect textural properties of extrudates.

Researchers have been used various terms such as common, regular, normal, and "wild-type" to differentiate ordinary corn from specialty corns. In the following discussion, the term *normal* corn will be used as the non-QPM genotype. QPM will be described later in this chapter.

Anatomy and chemical composition of the corn kernel

The corn kernel, classified botanically as a caryopsis, is the largest among the cereal grains, weighing an average of 250 to 350 mg (Johnson 2000). It contains

approximately 61-78% carbohydrates, 6-12% protein, 3-6% fat, 1-4% ash, 9-15% moisture, and 2-4% crude fiber (Watson 1987; Johnson 1991; Hoseney 1994). Four anatomical structures form the corn seed: endosperm, germ, tip cap, and pericarp or bran (Hoseney 1994). The endosperm, which accounts for almost 83% of the kernel dry weight, contains 74% of the total protein and 98% of the total carbohydrate, which is 86-89% starch. The germ, constituting 10-14% of the kernel weight, contains approximately 26% of the total protein, 83% of the fat, and the major amounts of vitamins, minerals, and sugar of the grain. The outermost structure of the seed, bran or pericarp, is 5-6% of the grain weight and contains 51% of the fiber found in the corn kernel. The tip-cap, a minor fraction, represents 0.8% of the seed weight and is composed basically of hemicellulose and lignin (Watson 1987; Johnson 1991; Johnson 2000).

Two endosperm patterns are present in maize: (1) horny/hard/vitreous and (2) opaque/soft/floury, due to the molecular and spatial arrangement of starch and protein in the endosperm cells (Hoseney 1994). In the horny or vitreous endosperm, the protein bodies, which are large in size, are embedded in a continuous proteinaceous matrix that is tightly packed inside the cells against the starch granules, which are polygonal in shape. The protein matrix surrounds the starch granules and encloses protein bodies, permitting few or no air spaces (Wilson 1987). In the floury endosperm, the central part of the corn grain, starch granules are spherical in shape and there are few protein bodies (Hoseney 1994; Larkins *et al.* 1995) within a discontinuous protein matrix around the starch granules. Due to this fact, the endosperm cells have air spaces that are believed to diffract and diffuse light, which makes corn appear opaque (Hoseney 1994).

Based on kernel characteristics, corn can be classified as flint, dent, flour, sweet, and popcorn (Watson 1987; Johnson 2000). Flint possesses a continuous volume of horny endosperm, which produces smooth and rounded kernels with a hard and vitreous appearance. Dent corn contains horny endosperm at the sides and the back of the kernel, while the central core is mainly soft and floury. In this type of corn, the soft endosperm is distributed up to the crown, which allows the formation of an indentation upon drying of the kernel. Flour type of maize also has a round crown but the endosperm is completely floury. Popcorn has a small flint type kernel with very hard, horn endosperm. Sweet maize results from *sugary* gene that prevents normal conversion of sugar to starch and the kernel accumulates phytoglycogen, a water soluble polysaccharide. This type of corn is primarily consumed as a vegetable in an immature stage (Hoseney 1994; Johnson 2000).

Starch

Starch is the most abundant component in the corn endosperm. It is composed of two structurally distinct glucan polymers, amylopectin and amylose, whose molecules are highly organized within the starch granules (White 1994). These insoluble granules are synthesized within the cellular organelle amyloplast by apposition (Hoseney 1994). Starch granule size in corn ranges from 5 to 30 μm and their shape varies within the corn endosperm, from polygonal to almost spherical (Thomas and Atwell 1999). Amylopectin, the predominant molecule in maize starch, is a highly branched polymer composed of α-1,4 linear glucose polymer connected by α-1,6-linked branch points (Biliaderis 1998). Generally, amylopectin molecules have molecular weights in the range of 1,000,000 to 3,000,000, although some are as large as 5,000,000 (White 1994). Conversely, amylose

consists basically of α-1,4 linked D-glucose polymers, which are occasionally branched, and have molecular weights around 50,000 to 200,000 (White 1994).

In normal corn, amylopectin accounts for 75% of the total starch, whereas amylose represents only 25% (Thomas and Atwell 1999). However, plant breeders have developed genotypes that contain starch with essentially 100% amylopectin (waxy) or much higher amylose content (high amylose or amylomaize) (Biliaderis 1998).

The orientation of molecular chains of amylose and amylopectin packed in the starch granules form both crystalline and amorphous regions (Eliason and Tahan 2001). Although researchers are still learning about the organizational structure of the starch, it is believed that smaller chains of amylopectin are situated very close together, which allows for strong interactions and formation of "clusters" that are radially oriented and result in crystalline regions (Cooke and Giddley 1992; Tester 1997). Amylose may be interspersed among the amylopectin chains in both crystalline and amorphous lamellae (Biliaderis 1998). Earlier studies related this arrangement to the birefringence when the granule is viewed under a polarized light, showing a black Maltese cross with its center at the hilum (White *et al.* 1990; White 1994; Tester 1997). The fact that starch is birefringent implies that there is a high degree of molecular orientation in the granule from the hilum toward the periphery (Donald *et al.* 1997), the overall spherulitic organization, but crystallinity is not a prerequisite for birefringence (Biliaderis 1998).

Gelatinization and pasting

Although the starch granules are made up of hydrophilic polymers, the starch granule itself is not soluble in water at room temperature (Thomas and Atwell 1999).

This hydrophobic characteristic is due to the semi-crystalline structure of the starch granule, and also to the hydrogen bonds formed between hydroxyl groups in the starch polymers (Eliason and Gudmundsson 1996). When starch is added to excess water at room temperature it absorbs just 30% of its weight in water through hydrogen bonding in a reversible process (Camire *et al.* 1990). Nevertheless, when water and heat are simultaneously applied the amount of absorbed water increases with disruption of the less ordered amorphous regions of the granule, allowing water to associate with free hydroxyl groups at a point where water uptake is irreversible. At this point birefringence begins to disappear, defining the onset of gelatinization temperature or T_o (Thomas and Atwell 1999; Eliason and Tathan 2001). Continuous heating causes the semicrystalline structure of the granular starch to be disrupted with loss of birefringence. This is marked by polymer chain mobilization and can be called glass transition or T_g (Camire *et al.* 1990). The heat causes hydrogen bonds to be disrupted and allows penetration of water into the granule causing it to swell considerably. Eventually a pattern indicative of a completely amorphous material is seen (Eliason and Gudmundsson 1996). The irreversible disruption of the molecular order within the starch granule with melting of the crystalline fraction is termed *gelatinization*.

As the starch slurry continues to be heated, amylose molecules tend to leach out of the granule and remnant granules continue to swell resulting in an increase in viscosity of the medium. This phenomenon is called *pasting* (Eliason and Gudmundsson 1996).

For regular cornstarch in excess water, gelatinization temperatures range from 61 to 72°C (Campbell *et al.* 1995; Lu *et al.* 1996; Pollak and White 1997; Krieger *et al.* 1998). Different botanical sources of starch or even starches from different corn

genotypes show different gelatinization temperature ranges (White *et al.* 1990; Sanders *et al.* 1990; Li *et al.* 1994; Campbell *et al.* 1995; NG *et al.* 1997; Thomas and Atwell 1999). These differences can be used to define functional properties of starches (White 1994).

Many parameters affect the gelatinization temperature and temperature range. Perhaps the most fundamental factor is the water, due to its role as a plasticizer for the starch crystallites. The presence of water may decrease the glass transition temperature of the starch, and consequently will affect the melting temperature of the starch crystalline (Eliason and Gudmundsson 1996).

Changes in viscosity in a starch medium are commonly determined through viscosity profiles generated on a Viscoamylograph or a Rapid Visco Analyser (RVA). Samples of starch are mixed with excess water, followed by heating in the instrument at a preset temperature and shear rate for a determined length of time. The increase in viscosity is measured as torque on the spindle and recorded in either Brabender units (BU) or Rapid Visco units (RVU) that reflect paste consistency (White 1994). Relative differences among botanical sources of starches can be assessed by keeping analytical conditions constant and plotting the profiles on a same graph (Thomas and Atwell 1999).

Differential scanning calorimetry (DSC) also has been extensively applied to study starch gelatinization properties (Stevens *et al.* 1971; Blanshard 1978; Krueger *et al.* 1987; Zeleznak and Hoseney 1987; Sanders *et al.* 1990; White *et al.* 1990; Campbell *et al.* 1994; Yamin *et al.* 1999; Eliason and Tathan 2001; Matveev *et al.* 2001; Seetharaman *et al.* 2001; Cassel 2002). This method is based on the specific heat and phase transitions of starch, which is related to changes in mobility of molecules and order of polymers during heating and cooling (White 1994).

Starch gelatinization is an endothermic process with enthalpy values in the range of 10-20j/g. (Eliason and Gudmundsson 1996). Thus, differences in thermal properties between a starch sample and a reference material sealed inside aluminum or stainless steel pans can be used to determine starch gelatinization properties (White 1994). The onset, peak, and conclusion temperatures of gelatinization and temperature of gelatinization range together with enthalpies of gelatinization can be precisely measured for different samples having their DSC-endotherm compared (White 1994; Thomas and Atwell 1999; Eliason and Tathan 2001).

Corn proteins

Researchers have used various procedures for isolating corn proteins, resulting in complex classification systems. Traditionally, maize seed proteins are sequentially fractionated into four major solubility classes according to Osborne (1924). The water-soluble fraction is *albumin*, while the proteins extracted with dilute salt solutions from the remaining residue are referred to as *globulins*. The *prolamin* fraction, which is also referred as zeins, is subsequently extracted with aqueous alcohol. The remaining insoluble proteins are referred to as *glutelins*, and these are extracted with dilute alkali or acid.

Landry and Moureaux (1970) included a reducing agent successively with aqueous alcohol, salt, and detergent to solubilize glutelins and separate them into three fractions. This led to five protein subsets in corn. Fraction I (FI), corresponded to albumins and globulins extracted from defatted corn meal with 0.5M NaCl. Fraction II (FII) was composed of zeins extracted with 55% isopropanol after the removal of

albumins, globulins, and salt. Fraction III (FIII) consisted of cross-linked zein and proteins close to zein in amino acid composition and was isolated in the presence of 55% isopropanol and a reducing agent (0.6% 2-mercaptoethanol). These proteins were alcohol-soluble glutelins called G1. Fraction IV (FIV), isolated in the presence of 0.5M NaCl and the reducing agent (2-mercaptoethanol) buffered at pH10, included proteins rich in basic amino acids, mainly histidine. They are also referred to as salt-soluble glutelins or G2. Finally, fraction V (FV) was referred to proteins isolated in the presence of a detergent (SDS 0.5%) and a reducing agent buffered at pH10, called true glutelins. An additional modification of this method by Landry and Moureaux (1976) allowed corn endosperm specific proteins to be regrouped into three major classes: zein, zein-like and prolamin-like.

Esen (1986) proposed a separation of zeins into three distinct classes, named alpha, beta and gamma-zeins, on the basis of the solubility of prolamins in 2-propanol in the presence or absence of a reducing agent. A different approach was taken by Wallace *et al.* (1990), who used borate buffer (pH 10) with a detergent (1% SDS) and a reducing agent (2% 2-mercaptoethanol) followed by addition of alcohol to 70% total volume to separate maize proteins into zein (sub classified as the α, β, γ and δ-zeins) and nonzein fractions. According to this classification, prolamins are referred to as zeins while albumins, globulins, and glutelins are referred to as non-zein proteins.

Albumins, globulins, and glutelins contain practically all the lysine and tryptophan of the maize kernel. Albumins and globulins encompass the majority of the enzymes present in the kernel and are synthesized early in kernel development (Murphy and Dalby 1971).

Zeins comprise more than 60% of the total protein of corn and 70% of the total endosperm protein in the normal corn (Hamaker *et al.* 1995; Landry *et al.* 2001). Consequently, they compose the major protein fraction of the corn kernel. Sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) analysis reveals that zeins are a mixture of polypeptides, the most predominant of which have molecular masses of M_r 50kD, 27kD, 22kD, 19kD,18kD, 16kD, 14kD and 10kD (Larkins and Lopes 1992; Woo *et al.* 2001). Based on their structure, instead of their differences in mobility or solubility, zeins can be classified into four groups (Coleman *et al.* 1999). The complex group of the closely related polypeptides of M_r 22kD and M_r 19kD are called α-zein (Esen 1986). The M_r 14kD protein is called β-zein, whereas the M_r 50kD, 27kD and 16 kD proteins are termed γ-zeins. The last ones, M_r 18kD and 10kD, are referred to as δ-zein (Lopes and Larkins 1992; Woo *et al.* 2001). In the normal corn genotype, the predominant zein proteins are the 22 kD and 19kD α-zeins and the 27kD γ-zein (Larkins *et al.* 1995).

Zein fractions are distinguishable in their amino acid sequence (Appendix II), chain size and spatial organization within the protein bodies (Esen 1986). α-Zein constitutes 75 to 85% of the total zein, and range in size from 210 to 245 amino acids. These polypeptides have high contents of glutamine and also leucine, alanine, and proline, which are predominantly hydrophobic amino acids (Shotwell and Larkins 1989). These proteins show a distinguishing feature such as a series of nine to ten tandemly repeated homologous peptides of 20 amino acids that interact to form alpha-helices with polar and hydrophobic residues facing outward and glutamine residues on the top and bottom. This organization causes the protein to fold into rod-shaped molecules (Argos *et al.* 1982). A three dimensional structure of this model was described by Matsushima *et*

al. (1997) using small-angle X-ray scattering. β-Zein, which represents 10-15% of the total zein, is 163 amino acids long and contains more methionine and cysteine, but lower levels of other essential amino acids than α-zeins.

γ-Zein accounts for 5-10% of the total zein and its 27kD polypeptide is a cysteine-rich (7%) fraction of 180 amino acids, containing also exceptionally high levels of proline (25%). The shortest chain is δ-zein of M_r 10kD, which contains 130 amino acids, and considerable amounts of the sulfur amino acids methionine and cysteine but has also significant contents of proline and leucine (Larkins and Lopes 1992). β- and γ-zein contain smaller amounts of leucine and other hydrophobic amino acids, and much higher amounts of cysteine than does α-zein. On the mole % basis, β-zein has about 5mol% cysteine, and γ-zein has 7mol%, whereas α-zein only contains 1mol% cysteine (Esen *et al.* 1981; Paulis 1981, Lopes and Larkins 1992).

Zein proteins are synthesized on rough endoplasmic reticulum membranes, and they associate within this organelle into insoluble accretions called protein bodies (Duvick 1961; Larkins and Hurkman 1978). In the endosperm, zeins have been described constrained to these spherical organelles (Lending and Larkins 1989) that start to accumulate in the endosperm of developing corn seed 10-25 days after pollination (Clore *et al.* 1996). Early in development, protein bodies are small in diameter (0.2 μm) and have both β and γ-zeins, but little or no α-zeins (Lending and Larkins 1989). In the final stages of protein body maturation, α-zeins fill most of the core of the protein body surrounded by a thin layer of β and γ-zeins (Dombrink-Kurtzman and Wilson 1992). As α-zeins penetrate the network of β and γ- zeins, it causes the protein bodies to expand to the diameter of 1 to 2 μm, which are mostly found in the internal regions of the

endosperm. In common corn, α-zeins are the most abundant fraction, followed by β and γ fractions, but only small amounts of δ-zeins are present in the protein bodies (Lending and Larkins 1989; Larkins *et al.* 1995).

Zeins contain no lysine and very small amounts of tryptophan, two essential amino acids, which are present in non-zein fraction (Lopes and Larkins 1992). Since zeins are predominant proteins in the maize endosperm, they define the protein quality of the corn protein. Consequently, maize protein is considered to be of poor nutritional quality having lysine and tryptophan, respectively, as the first and second limiting amino acids (Paes and Bicudo 1995).

Functional properties of zeins

Zeins contain significant amounts of glutamine, an amino acid that carries uncharged polar terminal groups and, therefore, promotes association of these proteins through hydrogen bonding. However, the presence of leucine, proline, alanine, and phenylalanine contributes to hydrophobicity of zeins, which defines unique functional properties of these proteins (Wall and Paulis 1978) and contributes to its ability to form film or fibers, important in multiple food applications (Lawton 2002).

Zein-based films provide gas and water impermeability as do ethyl cellulose and etherified cellulose (Beck *et al.* 1996) with the advantage of being a biodegradable packing material (Lai and Padua 1998). The water barrier property of zein was demonstrated to be useful in retarding moisture loss before popping in popcorn (Wu and Schwartzberg 1992) as well as in preserving coating nuts, dried fruits and candies (Wilson 1987). Another property for zein film, that of a fat barrier, was observed by

Mallikarjunan *et al.* (1997), who studied the effectiveness of zein as an edible coating on a fat-free starch product to reduce oil uptake during frying. Recently, it has been demonstrated that zein films allowed for the development of modified atmosphere packaging for broccoli florets, which maintained their original firmness and color up to 6 days when stored in glass jars sealed with zein film (Rakotonirainy *et al.* 2001).

Above its glass transition temperature (T_g), approximately 25°C, protein body-free zeins have also been shown to form viscoelastic networks similar to those of gluten when mixed in a starch dough system (Lawton 1992). Recently, the formation of zein aggregates has also been shown to be an important factor influencing textural parameters in ready to eat corn-based foods. Batterman-Azcona (1998) observed that addition of commercial zein to cornstarch, prior to extrusion, caused strengthening of the extrudates. Similarly, Tandjung (2000) has demonstrated that purified zein added to starch modified extrudates texture. Starch-zein extrudates showed higher brittleness and stiffness than cornstarch extrudates, after being conditioned to specific moisture levels.

Quality Protein Maize (QPM)

In 1964, Mertz and collaborators found mutant kernels of corn that had floury endosperm and high lysine content in the protein fraction (Mertz *et al.* 1964). The content of that amino acid was two-fold higher (4g/100g protein) in the mutant *opaque-2*, compared to common corn (2g/100g protein). Together with increased lysine levels, the researchers observed higher amounts of tryptophan, the second limiting amino acid in corn. Following the discovery of the genes responsible for the enhanced amino acid profile of corn protein, many biological trials were carried out in humans and animals to

evaluate the protein quality of the *opaque-2* materials *in vivo*. The superior protein quality of the *opaque-2* genotypes has been extensively reported in the literature (Mertz *et al. 1*965; Bressani 1966; Bressani *et al.* 1969a; Bressani *et al.* 1969b; Clark *et al.* 1967). Thereafter, plant breeders attempted to improve the protein quality of corn grain on a commercial scale. The transfer of either the gene *opaque*-2 or gene *floury*-2 was facilitated by simple and straightforward backcross schemes, observing the soft chalky phenotype in subsequent generations (Vasal 1994). By the early 1970s, high lysine corn was ready for commercial exploitation in different countries such as Brazil, Colombia, United States, Yugoslavia, South Africa and India (Mertz 1992).

In most instances, grain yield of o*paque–2* cultivars was disappointing and other undesirable agronomic characteristics existed, such as greater susceptibility to ear rot and stored grain pests (Magnavaca 1992). In developing countries, where farmers are familiar with growing hard flint and dent maize cultivars, soft materials were not well accepted either by producers or by processors. Also, the kernels of *opaque-2* varieties had poor germination and greater kernel breakage compared to normal corn (Villegas *et al.* 1992). Because of these unattractive properties, the production of high lysine corn was abandoned in the 1970s (Mertz 1992). In an effort to reduce the defects of *opaque*-2 maize, many scientists in different countries experimented with various approaches in the development of high protein quality corn germplasm (Magnava 1992; Villegas *et al.* 1992). The most promising method was the use of modifier genes, discovered by screening some regions of *opaque-2* kernels that were more vitreous than others and which also contained acceptable levels of essential amino acids in the protein (Paez *et al.* 1969). Lysine content in modified kernels was reported by Villegas *et al.* (1992) as 3.4%

of the protein, which was 70% higher than that found in common corn endosperm, and slightly lower than the amount of this essential amino acid present in the soft region of *opaque-2*. Tryptophan level in the vitreous region, 0.85% of the protein, was two-fold higher than the value observed in normal corn endosperm. According to Mertz (1992), soft endosperm materials contain an average of 1.0% of tryptophan in the protein.

CIMMYT, the International Center for Improvement of Maize and Wheat, at El Batan, Mexico, started to breed kernels with modified endosperm. The result was a high lysine corn with vitreous endosperm and a high grain yield, indistinguishable from that of normal maize, except for the superior protein quality. This material was given the name of *Quality Protein Maize* (QPM) (Villegas *et al.* 1992; Vasal 1994; Vasal 2001).

QPM kernel structure and chemical composition

Kernel structure and proximate composition of QPM cultivars have been reported to be similar to normal corn, except for a higher concentration of lysine and tryptophan in the protein (Wu 1992; Kibuuka 1995; Paes and Bicudo 1995; Martinez *et al.* 1996). The weight of the Brazilian QPM cultivars kernels average 300 mg (Guimarães *et al.* 1995), which is similar to that reported by Serna-Saldivar *et al.* (1990) in a QPM inbred line produced in the United States.

In QPM genotypes, despite the differences in the ratio of non-zeins to zein, the content of γ-zein is primarily the most significant difference between normal and QPM genotypes. In QPM, γ-zein concentration is two to two and a half-fold higher than in normal corn, and α- and β-zeins are one third of the levels of the wild corn genotype (Paiva *et al.* 1991; Larkins and Lopes 1992; Moro *et al.* 1995). Also, spatial distribution

of zeins within protein bodies differs from that observed in common corn (Larkins *et al.* 1995). These alterations are genetically controlled by modifier genes that produce smaller sized γ-zeins-rich protein bodies (average 1μm) that are present in higher number in the endosperm of QPM genotype (Paulis *et al.* 1992). The small protein bodies with high γ-zein protein content have been suggested to account for the vitreousness and hardness of the grain (Moro *et al.* 1995), but its role on chemistry of the corn kernel is still unknown. Consequently, the importance of these modified protein bodies to a food system is still poorly understood.

Food extrusion

Invented in 1797 by Joseph Baramah to produce lead pipes, extrusion technology has become an important tool in the food industry since the development of the single-screw food extruder in the 1930s (Harper 1981; Chinnaswamy 1993). Extrusion is defined as a process in which moistened, starchy, and/or proteinaceous materials are cooked, plasticized, and shaped in an extruder by a combination of shear, heat, and pressure (Harper 1981). This process is often described as a high temperature/short time (HTST) procedure because the material can reach high temperatures, while exposure time to high heat is often very short.

A food extruder is considered to be a bioreactor that transforms raw material into modified, intermediate, and finished products. During extrusion, the material to be processed is introduced into a feed port at a steady regulated rate. The flights on the screw transport the ingredients through a barrel in a drag flow, promoting mechanical shear at the same time that temperature is applied or generated. Macromolecule

configuration and conformation changes occur in the feed material as well as the interaction with water that softens and melts the solid ingredient forming dough with specific viscosity (Harper 1986; Harper 1989; Mitchell and Areas 1992; Frame 1994; Moore 1994; Bhatnagar and Hanna 1997). As the molten dough moves through the extruder, the pressure within the barrel increases, due to restriction caused by a die at the barrel discharge (Moore 1994). Boiling or flashing of water does not occur within the barrel, because the pressure exceeds the vapor pressure of the water at the extrusion temperature. As the material exits through the die, pressure suddenly decreases (Harper 1989). Flashing of the moisture occurs upon exit, which causes the material to expand. Air pockets are created when vapor expands. The temperature drop permits the solidification of the product, which retains air cells and maintains its expanded shape (Harper 1981; Harper 1989; Bhatnagar and Hanna 1997). Extrusion produces final products that are completely different in taste, texture, and nutritional quality than their initial ingredients (Harper 1989).

Extruders consist of two basic components, screw and die, which can be further subdivided into three process areas or sections: feed, transition, and metering (Harper 1981; Riaz 2000). The feed section conveys the material down the screw to the compression section, where the material changes from a granular or particulate state to an amorphous or plasticized state. In the metering section, which has the highest shear rate, the viscous dissipation of mechanical energy is large, and the temperature and pressure increase rapidly before the material reaches the die outlet (Harper 1989).

Two major types of extruders have been described in the literature, single-screw and twin-screw, according to the number of compression screws inside the barrel (Harper

1989; Frame 1999; Riaz 2000; Rockey 2000). Single-screw extruders also can be subdivided in cold-forming, high-pressure forming, low-shear-cooking, collet and high-shear-cooking extruders, based on the screw design (diameter, number of flights, height and shape, channel width, pitch number, and root diameter) as well as the temperature reached within the jacketed barrel (Riaz 1997; Frame 1999). On the other hand, twin-screw extruders consist of two parallel screws in a barrel and can be non-intermeshed, co-rotating, non-intermeshed/counter rotating, intermeshed/co-rotating and counter rotating (Harper 1981; Riaz 1997).

Depending on their design, extruders can perform various process tasks in food manufacture. The machines can be used to mix, knead, disperse, plasticize, gelatinize, texturize, dissolve, cook, melt, roast, caramelize, sterilize, dry crystallize, react, expand, fill, and shape (Frame 1999; Riaz 2000). These processing steps also can be combined by choosing specific extrusion parameters and thus simplify conventional process techniques and operations (Meuser and Wiedmann 1989).

Low-shear stress extruders, often referred to as forming extruders, are designed to minimize mechanical energy to impart cooking of the dough as in pasta production (Riaz 2000). Medium-shear extruders impart higher mechanical energy inputs than low-shear extruders, and moisture levels of its resulting extrudates are lower than the first type extruder (Riaz 1997). Most pet food, aquatic feed, and textured vegetable protein industries use these low shear type of extruders (Frame 1994; Riaz 2000). High-shear extruders convey a high level of mechanical energy that is converted to heat in order to cook a dough and produce puffed snack foods, pet foods, ready-to-eat (RTE) breakfast cereals, candy, crisp breads, pregelatinized flours, dried soup mixes, instant beverage

powders, crackers, wafers, and texturizated soy proteins among others (Frame 1994; Riaz 2000; Rokey 2000).

Importance of extrusion conditions on extrudate characteristics

Temperature, screw design, screw speed, feed rate, along with composition and moisture content of the feed material, are the most important determinants of extrudate physical and nutritional properties (Harper 1981). Extrudates should present specific expansion, density, color, texture, and mouthfeel, among other characteristics to be acceptable by consumers (Halek and Clark 1992; Moore 1999; Rokey 2000). Thus, these characteristics shall be carefully taken into account when developing a new extruded product.

Adequate expansion is a prime requisite of satisfactory direct expanded cereal-extruded products due to the fact that it is directly related to their crispiness and crunchiness (Ali *et al.* 1996). Additionally, cornflakes and expanded breakfast cereals that remain crispy after they contact milk (long bowl-life), and also crunchy savory snacks, are preferred by consumers (Gould and Densk 1996).

Puffing is due to the vaporization of super-heated water during extrusion cooking when the melted dough is forced to a die and the pressure drop causes water to flash, forming tiny cells (Park 1976). Biopolymers, such as those of starch or certain proteins, form a fluid melt at high temperature, resulting in a continuous phase, which binds together all the other particulate matter of the dispersed phases within the feed and help to retain the air bubbles formed during the extrusion processing (Guy 1994; Moore 1999). The amount of the polymers found in the continuous phase essentially determines the

extensibility of the bubble cell walls and the overall expansion of an extrudates (Mitchell and Areas 1992). Many tiny air bubbles inside the extrudate render a porous, expanded, sponge-like structure in the product. Highly expanded products such as savory corn curls have high expansion ratio values, low density (48-64g/l), and show porous structures with large air pockets or cells (Owusu-Ansah *et al.* 1984; Bhatnagar and Hanna 1997).

The influence of the raw material composition in the final extrudate physical properties has been widely described in the literature (Miller 1994; Riaz 1997; Guy 1999; Miller 1999, Moore 1999). In the case of cereal-based extruded products, amylose to amylopectin ratio has been considered the major factor influencing expansion. Chinnaswamy (1993) observed a higher expansion ratio for extruded native amylomaize materials, rich in amylose, compared to waxy cornstarch, rich in amylopectin. Also, the percent addition of amylose up to 50% to regular cornstarch, which contains an average of 25% amylose and 75% amylopectin, resulted in a better expansion ratio. Bhuiyan and Blanshard (1982) reported similar results for corn grits and corn flours evaluated in relation to extrusion quality. Samples with higher amylose contents were classified as satisfactory based on expansion behavior. It has been postulated that amylopectin is degraded to a greater extent than amylose during extrusion, causing reduction in viscosity of the molten dough inside the barrel, which results in reduced residence time, pressure and expansion (Colonna *et al.* 1989).

Moisture content is perhaps the single most important processing variable of the extrusion process affecting physical properties of extrudates (Riaz 1997; Miller 1999). It plays an important role affecting molten viscosity, and acting as a plasticizer for polymers such as starch and protein (Guy 1994). It also influences heat sinking, which in

turn affects specific mechanical energy (SME) input (Moore 1994), which is the amount of mechanical energy per unit mass of material extruded (Levine 1997).

Expansion increases with moisture content but decreases at the lower temperatures normally found with higher moisture levels (Miller 1994) because of the reduced viscosity and increased specific heat. Specific energies for direct expansion of cereals are as high as 0.08kWhr/kg with high shear, thus high-shear cooking extrusion is suitable for direct expansion (Miller 1994). Puffing depends on SME input, which is affected by the predominance of either extrusion moisture or extrusion temperature. Generally, the expansion ratio of starch-based extrudates increases with increasing temperature when water content is lower than approximately 19.5% (Colonna *et al.* 1989). Higher process temperatures increase cooking of the molten mass, reduce viscosity and promote expansion (Sutheerawattananonda *et al.* 1994). Furthermore, temperature generated within the barrel due to shear promotes changes in macromolecules (Mercier and Feillet 1975). It is generally accepted that extrusion cooking of starches or starchy cereals involves extensive disruption of starch granules by the gelatinization process followed by degradation of starch molecules at high temperatures (Gomez and Aguilera 1984; Camire 1990; Wasserman *et al.* 1992; Wen *et al.* 1992; Mitchell and Areas 1992; Miller 1999). This permits the formation of the homogeneous and flexible molten phase needed for expansion (Colonna *et al.* 1989; Chinnaswamy 1993). Several researchers demonstrated that expansion of extruded cereals or starch-based materials depends on the degree of either starch gelatinization or dextrinization (Gomez and Aguilera 1983; Bhattacharya and Hanna 1987; Lue *et al.* 1990). Samples do not start to expand until a die head temperature of approximately

100°C has been reached (Gomez and Aguilera 1984; Harper 1989). For corn grits, increases in barrel temperature results in an increase in the percent of gelatinized starch with a better expansion ratio (Bhattacharya and Hanna 1987). Under severe conditions such as barrel temperatures over 170°C, 20% moisture and 100 rpm screw speed, the granular structure of starch is destroyed and cell walls ruptures causing expansion to decrease (Gomez and Aguilera 1984). Increasing extrusion moisture content resulted in decreased expansion of cornstarch (Chinnaswamy *et al.* 1989), corn grits (Gomez and Aguilera 1984; Milford *et al.* 1996), corn meal (Barrett and Ross 1990), wheat flour (Sutheerawattananonda *et al.* 1994), wheat starch (Faubion and Hoseney 1982), and sorghum meals (Gomez *et al.* 1988) when processed in a single-screw extruder. Ilo *et al.* (1996) also reported a decreased radial expansion ratio for extruded corn grits with increasing feed moisture, when a twin-screw extruder was applied. Nevertheless, Sutheerawattananonda and coworkers (1994) observed that increased moisture content promoted longitudinal expansion of wheat flour extrudates that altered the shape of the products. The reduction in expansion ratio due to increasing water content in the feed material is believed to be due to a decrease in molten viscosity with consequential drop in pressure differential and residence time (Bhattacharya *et al.* 1986), which is related to less starch gelatinization (Bhattacharya and Hanna 1987).

Increases in screw speed promote power input, therefore affecting shear rate (Colonna *et al.* 1989). Higher screw speed, however, results in higher moisture content in the final extrudates because of the lowered residence time (Miller 1990). Therefore, the outcome of screw speed increases is not apparent.

Most recent papers have been devoted to the concept of specific mechanical energy (SME) to better describe the overall processing conditions during extrusion processing of foods, since SME is a calculated numerical value that incorporates data of torque, revolutions per min (RPM), and feed rate applied in the process (Levine 1997). Thus, SME allows extrusion to be a viewed independently of the type of extruder used, as long as extrusion temperatures, time, and shear rate are identified (van Lengerich 1990). Therefore, SME obtained for a product in a specific extruder can be applied to produce similar extrudates with another machine.

Effect of extrusion on proteins

Extrusion has a profound effect on the structures of proteins present in a food system (Camire 1990; Areas 1992; Mitchell and Areas 1992). The effect of shear, pressure, and high temperature inside the extruder barrel cause protein denaturation and promote association of polymers, which in turn can be disrupted by heat to form a melted phase together with other biopolymers such as carbohydrates and lipids (Areas 1992). Covalent bonds formation and non-covalent interactions can possibly occur at high temperature and shear conditions at the metering section or reactor zone resulting in the recombination of peptides and alignment of the denatured proteins in the direction of the melt flow (Areas 1992). However, the way that protein-protein interactions happen during extrusion is still unclear, and no specific mechanism has been proposed to date.

Most raw materials used in the extrusion processing of cereal-based foods, such as flour and grits, are not comprised only of starch but they also contain proteins that can

be structurally modified during the extrusion processing, thus influencing extrudate physical properties (Riaz 1997; Riaz 2000).

Extrusion at temperatures near 100°C has been shown to cause a decrease in solubility of proteins of semolina as well as changes in the molecular distribution of the proteins in a SDS-PAGE analysis (Ummadi *et al.* 1995). After extrusion, formation of low molecular weight peptides was observed with a simultaneous disappearance of high molecular weight peptides of albumins, which was related to protein depolymerization. Eletrophoretic patterns of gliadin and glutenin, however, showed an opposite trend, suggesting that those proteins were more likely to form aggregates during extrusion. Comparison of gels under reduced and unreduced conditions demonstrated that disulfide linkages played a major role in the structural changes of semolina proteins during extrusion.

Li and Lee (1996a) also observed changes in the solubility and molecular weight patterns of wheat proteins upon extrusion of wheat flour at die temperatures above 160°C. Protein solubility decreased dramatically after extrusion in all solvents tested. This was accompanied by a decrease in the intensity of bands of the gliadin (~30kD to 100kD) and glutenin (>100kD) regions. Moreover, the use of a SDS+2-ME solvent readily solubilized proteins from extruded samples, which demonstrated that decreased protein solubility in processed wheat flour might have been a result of aggregation through both disulfide bonds and hydrophobic interactions. The same authors also investigated the role of protein changes in the physical properties of the wheat extrudates in another study and concluded that disulfide bonds played an important role in the protein network holding together the structure of the final product. The addition of cysteine to the wheat flour

before extrusion caused a decrease in expansion ratio and the water holding capacity of extrudates. On the other hand, whiteness value, gumminess, cohesiveness, and oil-absorption capacity in extrudates increased. Extrudates also had reduced cell size and cell thickness when observed with a scanning electron microscope (Li and Lee 1996b).

More recent studies also have shown that the structure of protein bodies and the chemistry of maize zeins are modified during extrusion and that these modifications may affect the texture of corn-based extrudates (Batterman-Azcona 1998; Tandjung 2000).

Batterman-Azcona and Hamaker (1998) reported intensive disruption of protein bodies and α-zein release in commercially extruded cornflakes evaluated through transmission electron microscopy. In a second study, Batterman-Azcona *et al.* (1999a), using transmission electron microscopy coupled with immunolocalization, observed that extrusion of corn flour at SME ~100 kJ/kg in a single screw extruder, resulted in the disruption of protein bodies and release of α-zein, which appeared to fuse together and form protein fibrils when SME higher than 150 kJ/kg was applied. Extrusion of corn flour under harsh conditions (SME ~380 kJ/kg) promoted formation of a zein network in a similar way to that of commercial extruded cornflakes.

In a study using model systems, Batterman-Azcona (1998) also evaluated modifications during extrusion on the protein bodies and zeins from either corn gluten meal (CGM) or purified zein as they were added to commercial cornstarch to achieve protein levels of 5, 10, and 20% in the final raw material. Commercial zein was not encapsulated in protein bodies, which was different from the original zein spatial organization within protein bodies seen in corn gluten meal. Under mild and harsh extrusion conditions, commercial zein added to starch formed large protein aggregates,

while protein bodies were disrupted and zein formed fibrils when starch/corn gluten meal was processed under higher SME conditions (> 220 kJ/kg). Extrusion resulted in less extractable proteins as determined in extractability and electrophoresis tests. Formation of large zein aggregates in commercial zein/starch extrudates resulted in less extractable proteins as compared to proteins extracted from CGM/starch extrudates, even when solvent containing a reducing agent (2-mercapthoethanol) was applied. Still the addition of the reducing agent significantly increased the amount of proteins extracted from CGM/starch extrudates, indicating prevalence of disulfide-bonds in the CGM protein after extrusion. The addition of corn gluten meal to starch tended to weaken extrudates as measured by the three-point bending and Kramer shear cell tests, regardless of the energy input during processing. Contrariwise, commercial zein added at low levels enhanced the strength of extrudates. The authors noted, however, that the effect of CGM on extrudates texture also could be influenced by other components of the corn gluten meal, such as lipids.

Tandjung (2000) reported the effect of added zein encapsulated in protein body form, and crude corn oil to the texture of commercial corn starch extrudates equilibrated to 3, 12 and 22% moisture. Increasing zein concentration of the starch from 10 to 20% caused a slight increase in the Young's modulus, or modulus of elasticity, which correlates with stiffness of extrudates, of the starchy extrudates without affecting extrudate brittleness, when measured using the three-point bending test. Generally, increases in SME resulted in lower modulus of extrudates and addition of oil plasticizing effect was only observed at bone dry and intermediate moisture levels. Brittleness of zein/starch extrudates was higher than for the pure starch extrudates conditioned to 3 and

12% moisture levels as measured through a Warner-Braztler test. The enhanced extrudate moduli and brittleness due to zein addition was accompanied by an increase in the glass transition temperatures (T_g) of extrudates compared to starch extrudates as measured using dynamical mechanical thermo analysis (DMTA). Changes in the T_g were attributed to protein fibril network formation during extrusion and increase in molecular weight of the resulting protein polymer. Overall, zein seemed to promote crispness in starch-based extrudates equilibrated to low and intermediate moisture levels.

Although several studies have been already carried out to clarify the importance of endosperm corn proteins in corn grain hardness and vitreousness, the role that protein compositional differences plays on the physical properties of texturized corn-based products is still poorly understood. Considering that protein has been shown to have an important impact in extruded food systems, it is important to understand how differences in composition or spatial arrangement of corn endosperm proteins, such as observed in Quality Protein Maize (QPM), can influence changes in polymers and structures during extrusion processing. Therefore, the overall objective of this project was to investigate the importance of the high γ-zein content in QPM to chemical, physical and microstructural changes that occurs in zeins during extrusion and its impact on texture of intermediate corn-based extrudates. The specific objectives were:

1. To determine composition and pasting properties of isolated starches from QPM and normal corn grits.
2. To evaluate the zein composition in normal and QPM corn grits produced in Brazilian commercial cultivars.

3. To study the effect of zein composition and extrusion conditions, as set by specific mechanical energy (SME), on changes in the microstructural organization of α and γ-zeins present in QPM and normal corn grits. To investigate textural properties of normal and QPM corn extrudates processed under similar extrusion conditions.

The experiments were designed to test the overall scientific hypothesis that high γ-zein content in QPM provides different textural properties in corn-based extrudates as compared to normal corn.

The scientific hypotheses evaluated in specific chapters were:

1. Starch isolated from QPM and normal corn grits are not different in regard to gelatinization and pasting properties.
2. Higher content of the cysteine-rich γ-zein in protein bodies of QPM confers resistance to disruption during extrusion.
3. The lower hydrophobicity of γ-zein causes increased dispersion of zeins as they are released from the constraint of the protein bodies during extrusion.
4. Zein polymerization through disulfide bonding occurs in higher degree in QPM extrudates as compared to normal corn extrudates.
5. Increased polymerization of zeins during extrusion of QPM grits result in more brittle corn-based extrudates.

CHAPTER II

GELATINIZATION AND PASTING PROPERTIES OF STARCHES ISOLATED FROM QPM AND NORMAL MAIZE GRITS

ABSTRACT

Starch was laboratory-isolated from maize grits prepared from kernels of the Brazilian QPM (BR 473 and BR 451) and normal (BRS 3143 and D 670) cultivars in order to be evaluated for functional properties. Starch samples were analyzed for pasting profiles using the Rapid Viscoanalyzer (RVA), and for gelatinization thermal properties by applying differential scanning calorimetry (DSC). Starches from maize grits of various sources had similar moisture and carbohydrate contents but different residual protein ($p<0.05$). Residual protein was higher for the starch samples isolated from QPM BR 473 and BRS 3143 when compared to the other two maize cultivars. Significant differences were observed among means of onset gelatinization temperature (T_o), and peak gelatinization temperature (T_p) among starches isolated from different maize grits, although means of final gelatinization temperature (T_f) and enthalpy of gelatinization (ΔH) for these same samples were not different ($p>0.05$). The overall mean for T_o of normal maize (67°C) was slightly lower than that of the QPM samples (67.7°C). A

similar trend was observed for T_p. Despite the differences ($p<0.05$) peak gelatinization temperature (T_p) values were of a narrow range. Maize starch samples were not different in regard to pasting temperature, peak time, peak viscosity and breakdown ($p>0.05$). But they differed in regard to hot paste viscosity, set back, and cooling stage paste viscosity ($p<0.05$). Starch isolated from grits of the BRS 3143 tended to have greater hot paste viscosity (179.9 RVU) than that isolated from QPM BR 451 (169.8RVU) and the normal maize D-670 (163.7 RVU)($p<0.05$), but not from the sample isolated from QPM BR 473 (172.7 RVU)). Also, final paste viscosity was greater for starch sample isolated from the normal maize BRS 3143 (281.2 RVU) as compared to starch samples of the QPM BR 473 (259.6 RVU) and normal maize D-670 (255.3 RVU) ($p<0.05$). However, maize type was not a cause of variation for either hot paste or final viscosity ($p>0.05$). Under the conditions of this study it can be concluded that laboratory-isolated starch from QPM and normal maize grits are not different in regard to pasting properties, although cultivar is an important factor affecting hot paste viscosity and cool paste viscosity of maize starches. In addition, starch isolated from QPM and normal maize grits have gelatinization properties somewhat dissimilar. Starch from QPM grits has slightly higher onset (T_o) and peak gelatinization temperature (T_p) than starch isolated from normal maize.

INTRODUCTION

Physical and sensorial properties such as texture and mouthfeel of most extruded products are primarily driven by the composition of the raw materials, although

processing conditions are also determinants of extrudates properties (Harper 1981; Halek and Clark 1992; Ali *et al.* 1996; Riaz 2000). In the case of maize-based savory snacks and breakfast cereals, starch is the most important component in the raw material usually affecting physical properties of end products (Mercier and Feillet 1975; Bhuiyan and Blanshard 1982; Chinnaswamy and Hanna 1988; Chinnaswamy 1993; Moore 1994; Riaz 1997). Differences in composition of starches contribute to unique physicochemical properties in food systems, since starch functional properties, including gelatinization and pasting, depend mostly on amylose and amylopectin contents and organization within the starch granules (White 2001).

As a starch suspension is heated, the viscosity of the media increases and a gel is formed when this paste is cooled. The ability of starch to gelatinize and form a gel is of primarily importance in food systems. This property is affected by the source of the starch and also the processing (Chinnaswamy 1993; White 2001). During extrusion of starchy materials, usually applied in the production of breakfast cereals and snacks, the effect of mechanical and thermal energy dissipation at moisture contents below 30% cause the native starch granules to melt and large polymers to fragment, resulting in the formation of a plasticized amorphous melt (Gomez and Aguilera 1984; Harper 1992; Went *et al.* 1992; Chinnaswamy 1993; Brummer *et al.* 2002). The characteristic of the viscous melting phase defines the extent of expansion and the characteristics of the extrudate, since the differential pressure at the die exit causes the plasticized starch to expand forming foam-like structures (Harper 1992; Brummer *et al.* 2002). Amylose to amylopectin ratio has been shown to be an important parameter affecting the melting phase and, therefore, affecting expansion of the extrudates, which is related to quality

attributes of expanded products such as crispness (Mercier and Feillet 1975; Harper and Tribelhorn 1991). However, proteins, mainly the alcohol soluble fraction zein, have been recently shown to play a role in the structure and texture of maize-based extrudates (Batterman-Azcona 1998) and may alter crispness and bowl-life of cornflakes (Tandjung 2000), two important attributes affecting cornflake quality. In the model systems applied to study the influence of zein in textural properties of extrudates by Batterman-Azcona (1998) and Tandjung (2000), maize starch samples were added to either commercial zein or maize gluten meal. Other ingredients such as maize grits and maize flours, which are used in the large-scale production of maize-based extrudates, have not been evaluated for this purpose to date. Consequently, in order to validate a method for testing the effect of zein in the texture of maize-based extrudates using maize grits, it is relevant to ensure first that no basic differences in the starch functional properties are also observed.

Functional properties of starch isolated from QPM maize have not been described in the current literature. Therefore, the present study was designed primarily to determine gelatinization and pasting properties of starch isolated from QPM and normal maize grits.

MATERIALS AND METHODS

Sample identification

Two Brazilian semi-flint maize QPM genotypes, the white variety BR 451 and the yellow variety BR 473, and two semi-flint normal maize genotypes, the white hybrid Dekalb D670 and the yellow hybrid BRS 3143 were evaluated in this experiment. Maize was grown at the Brazilian Agriculture Research System (Embrapa) - Maize and

Sorghum Research Center, Sete Lagoas, Minas Gerais State, Brazil, during the 2001 growing season. Four replicates were obtained for each maize genotype that were produced following a completely randomized design in production field.

Maize grits preparation

Grains were dehulled and degermed with an industrial abrasive degerminator (Farsete, Sete Lagoas, MG, Brazil). Resulting flaking grits were milled in a dry milling plant (Moinho Jóia, Belo Horizonte, MG, Brazil) to coarse grits (800μm). Further grinding with an abrasive disc mill (Buhler-Miag, model MLI-204, Minneapolis, MN) provided smaller particle size grits (~550μm) that is typically used for the large-scale production of maize-based snacks.

Proximate composition

Prior to chemical analysis, samples of maize grits were ground to fine flour in a cyclone mill (Cyclotec 1093, Tecator, Hoganas, Sweden) to pass through a 0.5mm-opening screen and air dried at 60°C for 48 hr as recommended by the AACC (1995). Ground grits samples were analyzed for moisture, ash, crude fat, crude protein, and crude fiber, according to the recommended methods 44-15A, 08-01, 30-25, 46-13, and 32-10 (AACC 1995), respectively, whereas, total carbohydrate was calculated by difference. All measurements were done in triplicate. Protein content was calculated by using the nitrogen to protein conversion factor 5.7.

Starch isolation and quantification

Starch was isolated from grits using a modified method from Bank and Greenwood (1975). Grits were soaked for 48h in 2mM acetate buffer, pH 6.5, containing 0.01M mercuric chloride. Wet grits were rinsed with distilled water and homogenized in a blender for 3min with 200 ml of distilled water. The resulting suspension was filtered through a 63 μm-opening stainless steel screen (250mesh) and rinsed with distilled water several times until no whitish material indicating presence of starch remained on sieve. The remaining residue on the screen after washing step was blended with distilled water for 1 min and filtered. This step was repeated twice and the collected suspension of starch was let to settle in a 1000 ml beaker covered with plastic film for approximately 3 hr at room temperature (22°C ± 2°C) before supernatant was decanted. The settled material was transferred to centrifuge tubes and a double volume of toluene in 0.05 M sodium chloride (1:10 ratio v/v) was added for removal of protein and small sugars. Tubes were agitated at slow movement for 2 hr followed by centrifugation at 300g for 5min. The supernatant was discarded and the emulsion layer of denatured protein that formed at the toluene-aqueous interface was removed with a spatula. Starch remaining in the aqueous layer was washed again with toluene once with 2 hr agitation time and twice with 1 hr agitation to eliminate the proteinaceous emulsion layer on the surface of starchy material. Resulting starch samples were defatted with 85% methanol under agitation for 24 hr at room temperature and centrifuged at 300g for 5 min for solvent removal. Starch samples were transferred to petri dishes and air-dried at 40°C overnight. Dry samples were homogenized with a mortar and pestle to fine flour, which was stored in glass containers inside a desiccator until further analyses.

Starch samples were analyzed for moisture, ash, crude fat, and crude protein according to the recommended methods 44-15A, 08-01, 30-25, and 46-13 (AACC 1995), respectively, whereas, total carbohydrate was calculated by difference. Analyses were done in triplicate for each starch sample.

Differential Scanning Calorimetry (DSC)

DSC studies were performed using a differential scanning calorimeter with a thermal analysis data station (model DSC 2920, Thermal Analysis, Inc., Newcastle, Delaware, USA) following the modified protocol described from Campbell *et al.* (1995). Prior to the analysis, starch samples were equilibrated for moisture content in a desiccator containing phosphorus pentoxide (P_2O_5) at 25°C (Polio *et al.* 1996). Samples reached approximately 1% moisture content after 72 hr as determined by weight loss. Following equilibration, starch samples (4 mg, dry weight basis) were weighed into aluminum pans and distilled water was added in a 2:1 (v/w) ratio. Pans were immediately sealed and allowed to equilibrate at room temperature (25°C) for 4 hr. To ensure no moisture loss due to poor crimping, pans were weighed before being transferred to the DSC cell. The DSC thermal program consisted of 10 min isothermal stage at 15°C, and heating from 25 to 120°C at a rate of 10°C/min. An empty pan was used as reference and indium was applied for calibration. Onset temperature (T_o), peak temperature of gelatinization (T_p), final temperature of gelatinization (T_f), and gelatinization enthalpy (ΔH) were determined using the Universal v2.3c program (Thermal Analysis, Inc., Newcastle, Delaware, USA). Tests were done on four replicate samples.

RVA profiles

Pasting profiles of the cornstarch samples were studied in a Rapid ViscoAnalyser (RVA) Series 4 (Newport Scientific, Warriewood, NSW, Australia) using the standard STD1 temperature profile. Slurries were prepared by combining 3 g (dry weight basis) of starch and 25 ml of deionized water. Samples were stirred at 960 rpm for 10 sec to disperse the starch, and then the drive motor was switched to 160 rpm, which was maintained for the remaining time of the test. Samples were equilibrated at 50°C for 1 min and then heated at a constant rate of 12°C/min to 95°C, maintained at 95°C for 3 min. The temperature was decreased at a constant rate over 3 min to 50°C, where it was maintained until the end of the test. Tests lasted 13 min. Pasting temperature, peak time, peak viscosity, breakdown, trough peak viscosity, set back, and final viscosity were recorded with an interfaced computer with the software Thermocline for Windows (V 1.2) ((Newport Scientific, Warriewood, NSW, Australia). Measurements were done in duplicate for each starch sample and values averaged.

Experimental design and data analysis

The experiment was set as a completely randomized design with four treatments (maize cultivars) and four replicates, which corresponded to samples of grains for each maize cultivar harvested at Embrapa (Sete Lagoas, MG, Brazil). Data for chemical and rheological assays were analyzed using ANOVA and subsequent LSD test was applied for mean treatments comparison at $p<0.05$. For comparison of means within maize type (QPM and normal) the contrast test was used, whenever significance differences were detected among cultivars. The software used for data analysis was SYSTAT 8.0 (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Proximate composition

Proximate composition of the various maize grits used in this study is presented on Table 2.1. As expected, carbohydrates were the major component of the grits (83.3 to 84.9%), followed by protein (5.8 to 6.9%), and crude fat (0.3 to 1.1%). Despite the significant differences in composition of the maize grits, values were similar to the ranges reported for the endosperm of normal maize (Watson 1987).

Table 2.1
Proximate composition of maize grits obtained through dry milling of kernels of different QPM and normal maize cultivars

Maize type	Cultivar	%					
		Moisture	Carbohydrate	Protein (%N x 5.71)	Crude fat	Crude fiber	Ash
Normal							
	BRS 3143	8.4 [b]	83.3 [c]	6.9 [a]	0.9 [b]	0.2 [a]	0.3 [b]
	D-670	8.0 [b]	84.9 [a]	6.5 [b]	0.3 [d]	0.2 [a]	0.1 [c]
QPM							
	BR 473	8.2 [b]	84.1 [b]	5.8 [c]	1.1 [a]	0.4 [a]	0.4 [a]
	BR 451	9.3 [a]	83.6 [b]	5.9 [c]	0.7 [c]	0.3 [a]	0.3 [b]
	SEM	0.15	0.18	0.13	0.08	0.04	0.04

* Values are means of four replicates of maize grits.
** Means followed by a same small letter superscript within a column are not significant different ($p < 0.05$).
***LS Mean compared through LSD test.

In general, QPM grits had lower protein contents than grits of their normal counterparts ($p<0.05$) but this trend was not found for the other nutrients. Grits of the normal maize D670 had slightly higher carbohydrate content (84.9%) than the normal BRS 3143 (83.3%) ($p<0.05$) but there were no differences in carbohydrate content within QPM samples ($p>0.05$). Grits differed in total crude fat ($p<0.05$) with the highest value for the QPM BR 473 (1.1%) and the lowest (0.3%) for the D-670. This trend was also observed for the ash content in maize grits. Crude fiber contents were similar for the various maize grits ($p>0.05$).

Starch composition

Results for chemical analyses of the isolated starch samples are presented in Table 2.2. Lipids and ash contents on starch samples were negligible. Therefore, they were omitted in further discussion of results.

Starches from various maize grits presented similar moisture and carbohydrates ($p>0.05$) contents but different residual protein ($p<0.05$), which was higher for the QPM BR 473 and BRS 3143 compared to the other two samples. Since the elimination of the protein during the starch isolation procedure is done by scraping out the emulsion layer of proteinaceous material, small differences in protein in the starch samples would be expected. Residual protein contents near 1% have been found elsewhere for laboratory-isolated maize starch (White *et al.* 1990).

Table 2.2
Composition of starches isolated from normal and QPM maize grits

Maize type	Cultivar	% Moisture	% Carbohydrates	% Protein
Normal				
	BRS 3143	9.83 ± 0.40	89.98±0.29	1.19±0.11 a
	D-670	8.61±0.08	90.52±0.03	0.88±0.05 b
QPM				
	BR 473	8.94±0.26	90.01±0.26	1.05±0.00 a
	BR 451	8.61±0.27	90.55±0.28	0.85±0.01 b

* Values are means of four replicates of maize grits.
** mean ± SEM
** Means followed by a same letter within a column are not significant different ($p < 0.05$) as compared applying the LSD test.

Thermal analysis by differential scanning calorimetry (DSC)

DSC analysis was carried out to evaluate the thermal behavior during starch gelatinization in excess water as an indicator of functional properties of the starches. The resulting data are presented in Table 2.3.

Significant differences were observed among means of onset gelatinization temperature (T_o), and peak gelatinization temperature (T_p) among starches isolated from different cultivars, although final gelatinization temperature (T_f) and enthalpy of gelatinization (ΔH) means for the same samples were not different ($p>0.05$).

Means for T_o ranged from 66.4 to 68.1°C, with the highest value for the starch isolated from QPM BR 451 grits and the lowest for the starch sample isolated from the normal maize BRS 3143. The overall mean of T_o for normal maize (67°C) was slightly lower than that of the QPM samples (67.7°C). Peak gelatinization temperature (T_p) values

presented a narrow range, despite the significant differences. Similar to the results for onset gelatinization temperature, T_p was higher for the QPM group (71.9°C).

Table 2.3
Thermal analysis of gelatinization of starch samples isolated from QPM and normal maize as determined by differential scanning calorimetry (DSC)

Maize type	Cultivar	Thermal properties			
		T_o(°C)	T_p (°C)	T_f (°C)	ΔH (J/g)
Normal	BRS 3143	66.4 ± 0.47^{c}	70.8 ± 0.07^{d}	82.5 ± 0.54^{a}	4.18± 0.19^{a}
	Dekalb D 670	67.6 ± 0.52^{b}	71.9 ± 0.12^{b}	82.9± 0.25^{a}	3.84± 0.09^{a}
	mean	67.0 ± 0.19 ^{B}	71.4 ± 0.16 ^{B}	82.7± 0.29 ^{A}	4.01± 0.11 ^{A}
QPM	BR 473	67.4 ± 0.13 ^{b}	71.6 ± 0.09^{c}	82.4± 0.37^{a}	3.89± 0.11^{a}
	BR 451	68.1 ± 0.26^{a}	72.3 ± 0.16^{a}	83.5± 1.14^{a}	4.02± 0.26^{a}
	mean	67.7± 0.13 ^{A}	71.9 ± 0.11 ^{A}	82.9 ± 0.3 ^{A}	3.96 ± 0.07 ^{A}

*Results are the average of four replicates for each starch sample. T_o= onset gelatinization temperature, T_p = peak gelatinization temperature, T_f = final or conclusion gelatinization temperature, ΔH = enthalpy of gelatinization.
**Values followed by the same superscript (small or capital letters) within a column are not significantly different at p>0.05.

Data found in this study for T_o, T_p, T_f and ΔH are somewhat similar to those previously reported by other authors for starch isolated from normal maize genotypes. Krueger *et al.* (1987) found similar value for T_f(70.3°C), but lower values for T_o (63.9°C) and ΔH (2.61) cal/g, respectively. Li *et al.* (1994) have shown ranges for T_o, T_p and T_f of 64.3-69.6°C, 70.1-73.9°C and, 76.8-79.6°C, respectively, for laboratory isolated starch from 35 tropical and semi-tropical maize populations. They also reported enthalpy of gelatinization range of 8.2-12.3J/g with mean of 10.5J/g.

Table 2.4
Pasting properties of starch isolated from grits of Brazilian QPM and normal maize cultivars evaluated through the RVA

Maize type	Cultivar	Pasting parameters						
		Pasting temperature	Peak time	Peak viscosity	Breakdown	Trough or hot paste viscosity	Setback	Final or cool paste viscosity
		(°C)	(minutes)	RVU				
Normal	D-670	75.3[a]	4.5[a]	283.8[a]	120.1[a]	163.7[b]	91.6[ab]	255.3[b]
	BRS 3143	74.9[a]	4.6[a]	289.1[a]	109.2[a]	179.9[a]	101.3[a]	281.2[a]
QPM	BR 451	75.5[a]	4.5[a]	289.9[a]	120.1[a]	169.8[b]	101.5[a]	271.3[ab]
	BR 473	75.3[a]	4.6[a]	279.5[a]	106.8[a]	172.7[ab]	86.9[b]	259.6[b]
	SEM	0.006	0.036	3.4	3.2	2.1	9.5	3.5

* Values are means of four replicates.
** Means followed by same letter within a column are not significantly different at $p < 0.05$ according to the LSD test

characterized by starch granules fully swollen and intact. By holding the high temperature, the effect of shear causes the starch granules to disrupt accompanied by molecular alignment of solubilized molecules, usually amylose, which causes a drop in the viscosity of the paste (breakdown). The drop in viscosity reaches a stable point, which is termed trough or hot paste viscosity. By reducing the temperature applied to the paste and maintaining the shear, amylose molecules start to reassociate and cause an increase in viscosity (setback).

The maize starch samples evaluated in this study were not different in regard to pasting temperature, peak time, peak viscosity, or breakdown ($p>0.05$). But they did differ in regard to hot paste viscosity, set back, and cooling paste viscosity within cultivars ($p<0.05$) (Table 2.4). Starch isolated from grits of the BRS3143 tends to have greater hot paste viscosity than starch isolated from QPM BR 451 and the normal maize D-670 ($p<0.05$), but did not differ from the sample isolated from QPM BR 473 (Table 2.4). Final paste viscosity was greater for the starch sample isolated from the normal maize BRS 3143 as compared to starch samples of the QPM BR 473 and normal maize D-670 ($p<0.05$). Consequently, values for setback, which is the difference of final viscosity minus trough viscosity, were smaller for the starch sample of the BR 473 as compared to samples of QPM BR451 and BRS 3143. Nevertheless, when means for cool paste viscosity, setback and hot paste viscosity were analyzed by maize type, initial significant differences within cultivars were no longer perceived for those variables (Table 2.5) ($p>0.05$).

Morphological and molecular changes that have been described for starch granules and their components during extrusion are somewhat different from those found

in a system with excess water such in the RVA and the DSC analysis applied to evaluate functionality of starch samples. In the extrusion of maize-based materials to produce breakfast cereals and snacks, usually low moisture (<30%) and high temperatures (>120°C) are applied (Riaz 2000; Moore, 2001).

Table 2.5
Hot paste viscosity, setback, and final paste viscosity values from RVA analysis of starch samples isolated from maize grits by maize type

Maize type		Trough viscosity	Setback	Final viscosity
			RVU	
Normal		171.3 [a]	96.47 [a]	265.5 [a]
QPM		172.9 [a]	94.19 [a]	270.1 [a]
	SME	2.11	3.80	3.52

* Values are means of eight replicates within maize type group.
**Means followed by same letter within a column are not significant different at $p < 0.05$ according to the contrast analysis.

These conditions have been shown to promote not only gelatinization but also disruption and melting of the starch granules accompanied by dextrinization of dispersed molecules, mainly amylopectin (Gomez and Aguilera 1984). Therefore, different states can be found in the molten dough during extrusion of starchy materials, gelatinized and fragmented granules, and dextrinized large fragments (Gomez and Aguilera 1984; Harper 1992). For that reason results from the RVA analyses might not correlate to that of the extrusion processing and changes in viscosity of the plasticized molten starch formed during extrusion may not be predicted by the results of the RVA profiles.

CONCLUSIONS

Under the conditions of this study, it can be concluded that isolated starch from QPM and normal maize grits are not different in regard to pasting properties, although cultivar is an important factor affecting hot paste viscosity and cool paste viscosity of maize starch. In addition, starch isolated from QPM and normal maize grits have thermal gelatinization properties somewhat dissimilar. Starch of QPM grits had slightly higher onset (T_o) and peak gelatinization temperature (T_p) than starch isolated from normal maize. However, overall results of starch thermal and pasting properties show either no or negligible differences between starch of normal and QPM cultivars. This indicates that further study of protein microstructural changes and differences in grits extrudate functionality, reported in Chapters III and IV, are more likely due to documented differences in zein proteins in normal and QPM cultivars.

CHAPTER III

MICROSTRUCTURAL MODIFICATIONS IN PROTEIN BODIES, ALPHA, AND GAMMA ZEINS DURING EXTRUSION OF QPM AND NORMAL MAIZE GRITS

ABSTRACT

Chemical and physical modifications in the maize prolamins, zeins, occur during extrusion and these changes have been related to final texture of extruded corn-based products. Despite the understanding of the importance of protein composition to molecular changes of protein during thermal processing in the production of foods, differences in zein composition, such as the higher γ-zein content reported for the specialty corn Quality Protein Maize (QPM), have not been exploited regarding its effect on physical and chemical modifications of protein bodies and zeins in the production of maize extrudates. Therefore, the objectives of this study were to evaluate the effect of extrusion on the organization of protein bodies and zeins in QPM and normal maize grits, and to investigate the influence of protein composition on microstructural changes of protein bodies in QPM and normal maize grits as a result of extrusion processing. Maize grits were produced with kernels of the Brazilian QPM cultivar (BR 473) and the normal maize cultivar (BRS 3143). Raw maize grits were evaluated for zein composition through

densitometry and for structural pattern using scanning electron microscopy. Grits were processed in a single-screw extruder at five different levels of specific mechanical energy (50, 75, 160, 260, 320, and 430kJ/kg). The progressive structural transformations of the major components of the maize grits as a result of the extrusion were investigated in cross sections of the extrudates using transmission electron microscopy with immunolocalization of α- and γ-zeins. Each of the three major components identified in the raw grits, starch granules, protein matrix, and protein bodies, developed independent structures during extrusion at the different levels of shear. Starch granules gelatinized and formed a continuous matrix, whereas, proteins aggregated into large masses diffused in the gelatinized starchy network. The high γ-zein content in QPM seems to confer resistance to disruption of protein bodies during extrusion. Therefore, high γ-zein rich protein bodies require higher energy input within the extruder for complete disruption than protein bodies found in normal maize. Differences in dispersion of zeins in the system for the two maize types were not identified at high shear conditions evaluated in this study.

INTRODUCTION

During the extrusion of cereal-based materials for the production of snack foods, breakfast cereals and petfood, biopolymers present in the feed material, such as starch and protein, are subjected to a combination of pressure, heat, and mechanical shear within the barrel of the extruder (Harper 1989). This condition creates many opportunities for

modification in the original structure of these polymers (Camire 2000). Starch granules lose their orderly organization and gelatinize (Gomez and Aguilera 1984), and can undergo fragmentation when temperatures above 150°C and high screw speed are applied in the extrusion processing of cereal-based materials (Wen *et al.* 1990; Wasserman *et al.* 1992). The gelatinized and fragmented starch forms a continuous melted phase, which binds other particulate matter of the dispersed phase and favors the expanded final product (Guy 1994). Proteins in the flour disaggregate through mechanical mixing, forming a homogeneous suspension as they denature, dissociate and unravel (Areas 1992). Denatured protein can cross-link, forming large aggregates as disulfide bonds break and reform, at the same time that new electrostatic and hydrophobic interactions may occur (Areas 1992; Li and Lee 1996). Therefore, molecular changes in proteins are mainly driven by their primary structure, dictated by the amino acid sequence, residues sequence and molecular weight (Sikorski 2001).

Despite the predominant content of starch in degermed maize meal, maize grits and maize flours, ingredients used in the production of expanded snacks and breakfast cereals (Riaz 1997), protein is also an important component of these materials (Guy 1994). In the normal corn, zeins, the maize prolamins, comprise more than 70% of the total endosperm protein (Hamaker *et al.* 1995; Landry *et al.* 2001) and are the principal protein source in maize. Those proteins are a mixture of polypeptides, the most predominant of which have molecular masses of M_r 50kD, 27kD, 22kD, 19kD, 18kD,16kD, 14kD, and 10kD (Larkins and Lopes 1992; Woo *et al.* 2001). Based on their structure, instead of their differences in mobility or solubility, zeins are classified in four groups (Coleman *et al.* 1999). The complex group of the closely related polypeptides of

M_r 22kD and M_r 19kD are called α-zein (Esen 1986). The M_r 14 kD protein is titled β-zein, whereas the M_r 50kD, 27kD, and, 16 kD proteins are termed γ-zeins. The last ones, M_r 18kD and10kD, are referred to as δ-zein (Lopes and Larkins 1992; Woo *et al.* 2001).

Zein fractions are distinguishable by amino acid sequencing and chain size (Esen 1986). α-Zein ranges in sizes from 210 to 245 amino acids and has high contents of glutamine, leucine, alanine, and proline, which are predominantly hydrophobic amino acids (Shotwell and Larkins 1989). Its structure is predicted to be an alpha helix that is arranged in a rod-shape molecule (Garratt *et al.* 1993). Contrary to α-zeins, γ-zein 27kD polypeptide is a cysteine-rich (7%) fraction of 180 amino acids, containing also exceptionally high levels of proline (25%). However, the γ-zein 50kD is a histidine-rich fraction (Woo *et al.* 2001). β-Zein is composed of 163 amino acids and contains more methionine and cysteine, but lower levels of other essential amino acids than α-zeins. The shortest chain is δ-zein, which contains approximately 130 amino acids and, is exceptionally rich in the sulfur amino acids methionine and cysteine (Chui and Falco 1995). It contains significant amounts of proline and leucine and is considered the most hydrophobic zein fraction (Woo *et al.* 2001). β- and γ-zeins, which are closely related, have smaller amounts of leucine and other hydrophobic amino acids, and much higher amounts of cysteine than does α-zein (Esen *et al.* 1981; Paulis 1981, Lopes and Larkins 1992).

In the endosperm, zeins are constrained to the membrane bound spherical organelles that are embedded in the protein matrix that surrounds the starch granules (Lending and Larkins 1989; Chandrashekar and Mazhar 1999). The fractions differ in their spatial organization within the protein bodies (Esen 1986). In the final stages of

protein body maturation, α-zeins and small amounts of δ-zein fill most of the core of the protein body surrounded by a thin layer of β and γ-zeins (Dombrink-Kurtzman and Wilson 1992). As α-zeins penetrate the network of β and γ- zeins, it causes the protein bodies to expand to the diameter of 1 to 2 μm. These protein bodies are mostly found in the internal regions of the endosperm of the mature maize grain (Larkins *et al.*1995). In normal maize, α-zein is the most abundant fraction, followed by β and γ fractions, but only small amounts of δ-zein is present in the protein bodies (Lending and Larkins 1989; Larkins *et al.* 1995). Nevertheless protein bodies in the specialty corn Quality Protein Maize (QPM) contain reduced amounts of α-zein and elevated content of γ-zein (Larkins *et al.* 1995). Concentration of γ-zein in QPM is two to three-fold higher than in normal maize, whereas α- and β-zeins are one third of the levels of those in the normal maize genotype (Paiva *et al.* 1991; Geetha *et al.* 1991; Larkins and Lopes 1992; Moro *et al.* 1995). Also, γ-zeins rich protein bodies are smaller size (average 1μm) and are present in higher number (Paulis *et al.* 1992). The small protein bodies with high γ-zein protein content have been suggested to account for the vitreousness and hardness of the grain (Moro *et al.* 1995), but its role on chemistry of the maize kernel and its importance to a food system is still poorly understood.

A microstructural evaluation of commercial extruded cornflakes using electron microscopy, coupled with immunolocalization of α-zein, revealed that the original round shape of protein bodies was lost during extrusion as they fused. Also dispersed zeins merged together to form a continuous network or fibrils. On the other hand, it has been demonstrated that protein bodies remain intact with their original spherical shape as corn flour is submitted to cooking in excess water only (Batterman-Ascona and Hamaker

1998). These data demonstrated the effect of shear and pressure within the extruder to disruption of protein bodies and release of zeins during extrusion of normal corn flour.

Different levels of specific mechanical energy (SME), defined as the amount of energy per material mass of material extruded (Levine 1997), have been applied to observe the effect of different levels of shear on changes in the microstructure of particulate material such as corn flour during extrusion. Batterman-Ascona *et al.* (1999a) evaluated microstructural changes in zeins in corn flour extruded in a single-screw extruder over a range of SME and concluded that disruption of the corn protein bodies occurs at SME of approximately 100kJ/kg. The authors also demonstrated that protein bodies were completely disrupted and α-zein was dispersed within the gelatinized starch matrix in the corn flour extruded at SME above 150kJ/kg. In addition, the authors observed aggregation of zeins in extrudates produced at levels of shear above 300kJ/kg. Variations of SME produced changes in texture in corn-based breakfast cereals.

Considering that protein composition is a major factor affecting its functionality, differences in proportion of the zein fractions, such as the one described for quality protein maize genotypes, may interfere in the physical modifications of protein bodies and chemical changes in zeins during extrusion. The high content of the cysteine-rich γ-zein in protein bodies of QPM corn may provide resistence to disruption during extrusion. As a result, more energy input would be required to fully break them and promote dispersal of zeins during extrusion of QPM maize grits. But once disrupted, dispersion of zeins might occur to a higher extent for QPM, followed by higher protein aggregation, because of the high content of sulfur amino acids in γ-zein. Therefore, the objectives of this study were to evaluate the effect of extrusion on the organization of

protein bodies and zeins in QPM and normal maize grits and, to investigate the influence of protein composition on microstructural changes of protein bodies in QPM and normal maize grits as a result of extrusion processing.

MATERIALS AND METHODS

Materials

Brazilian maize genotypes *QPM BR 473* and normal *BRS 3143* were grown at Embrapa Maize and Sorghum Research Center in Sete Lagoas, Minas Gerais, Brazil, during the 2001 growing season. Grains were dehulled and degermed with an industrial abrasive degerminator (Farsete, Sete Lagoas, MG, Brazil). Resulting flaking grits were milled in a dry milling plant (Moinho Jóia, Belo Horizonte, Minas Gerais State, Brazil) using roller mills, followed by sifting and aspiration to provide coarse grits (~800μm). Further grinding with an abrasive disc mill (Buhler-Miag, model MLI-204, Minneapolis, MN) provided smaller particle size grits (~550μm). Grits were analyzed for moisture content according to method 44-19 (AACC 1995), and a calculated volume of distilled water was added to each sample to reach a final moisture content of 22%. Samples and added water were mixed thoroughly for 20 min inside plastic bags for homogenization and, were allowed to moisture equilibrate for 24 hr at 10°C. To ensure the desired final moisture content in the samples, moisture was determined in the equilibrated samples following the method previously described. Conditioned samples were allowed to reach ambient temperature (~22°C) before being extruded.

Extrusion

Extrusion work was carried out at the facilities of the UDSA/ARS/NCAUR, Peoria, IL, with the assistance of Dr. John W.Lawton. Each sample of maize grits was extruded in duplicate with a Brabender plasticorder single-screw extruder, PL2000 (Type 832500 C.W. Brabender Instruments, Inc., South Hackensack, NJ) with barrel diameter of 19mm, length to diameter ratio (L/D) of 30:1, and a die nozzle diameter of 3mm. Three screws with different compression ratios (1:1, 3:1 and dispersive) were combined with different screw speeds (20, 40, and 60 RPM) to provide different shear within the barrel, which resulted in six different levels of specific mechanical energy (SME) (50, 75, 160, 260, 320, and 430 kJ/Kg). Temperature was maintained constant with electrically heated, compressed air-cooled collars around the barrel in order to reach desirable levels of SME. Temperatures were 120, 160, 120, and 100°C, respectively, along the three zones of the extruder barrel (T1, T2, and T3) and the die opening (T4). Sample feed rate was determined during each individual run using the weight (g) of the extruded material collected for a period of 30 sec in the middle of the run. A die was used to identify the starting point of sample collection for feed rate determination and also to identify and separate samples of extrusion runs. The order of processing was randomly chosen and each extrusion run was brought to steady state as indicated by constant torque, before sampling and data collection. Torque (Nm) and screw speed (rpm) were recorded during processing on an interfaced computer for automatic data collection, with the Basic Program with Multiple Evaluation (version 4.0.2) (Brabender Instruments, Inc., South Hackensack, NJ). SME was calculated by dividing the net power input to the screw by the extrudate feed rate following the formula:

$$\text{SME (kJ/Kg)} = [\text{Torque (Nm)} \times \text{screw speed (rpm)} \times 2\pi]/ \text{feed rate (Kg/hr)}$$

Resulting extruded rods were randomly collected in the air-cooling tunnel, and cut into approximately 20-cm length sample. Extrudates were air-dried at 40^0C to final moisture of approximately 9%, cooled at ambient temperature, and stored in sealed plastic bags at –20°C until texture analysis.

Quantification of α and γ-zeins in the grits

The content of α and γ-zeins present in the maize grits was measured through densitometry. Maize grits were ground using a cyclone mill (Cyclotec 1093, Tecator, Hoganas, Sweden) with a 0.5mm screen before analysis. Zeins were extracted from raw samples using the procedure described by Wallace *et al.* (1990) and subjected to SDS-PAGE on a vertical mini gel system (Mini-PROTEAN II cell, Bio Rad, Hercules, CA) using a polyacrylamide 12.5% (w/v) separating gel and a 4% (w/v) stacking gel, following the method described by Laemmli *et al.* (1970). The wells of the gel were loaded with samples of same protein content and there were two lanes for each normal and QPM maize types. The Dalton marker VI (Sigma Chemical Co., St. Louis, MO) was used as molecular marker. Electrophoresis was performed at 100 volts for 100 min. Gel staining was done with 0.025% Coomassie Blue R-250 in 50% ethanol and 10% acetic acid and destained in 10% acetic acid and 5% ethanol. For quantification of α and γ-zeins, the resulting gel was scanned in a personal densitometer model Molecular Dynamics SI (Amersham Biosciences, Sunnyvale, CA) and area of the bands analyzed using a computer interfaced to the densitometer running the software image QuaNT version 4.2 (Molecular Dynamics, Sunnyvale, CA). Triplicate measurements were taken for each band and values averaged for statistical analysis.

Scanning electron microscopy (SEM)

Raw maize grits were sprinkled onto labeled stubs previously covered with carbon conductive tape and coated with 20nm of gold using a sputter coater Emitech K550 (Emitech Ltd., Ashford, Kent, England) set to thickness. Specimens were examined in a digital scanning electron microscope Zeiss DSM 940 (Leo Electromicroscope GmbH, Germany) with an accelerating voltage of 15kV and emission current of 10 μA.

Transmission electron microscopy (TEM)

Raw and extruded samples were analyzed for possible physical changes in protein bodies and dispersion of the zeins due to processing by transmission electron microscopy (TEM) coupled with immunocytochemical labeling (IEM). Samples were cross sectioned into pie shape pieces, which were fixed in 1% glutaraldehyde, 4% paraformaldehyde in 0.1M potassium phosphate buffer, pH 6.8 for 16hr at 4°C. After removal of the fixative with 0.1M phosphate buffer, samples were dehydrated in ethanol-graded series, 15 min each, in 10, 30, 50, 70, 90, and 100%. After an additional 20 min in fresh 100% ethanol, samples were progressively infiltrated in LR White resin (London Resin Company Ltd., Reading, Berkshire, England) in absolute ethanol, 16 hr each, in 25, 50, and 75% resin. Samples were further embedded for 16 hr in 100% fresh resin, which was exchanged for fresh aliquots two additional times in 16 hr intervals. Specimens were placed at the bottom of flat-bottom embedding capsules (Electron Microscopy Sciences, Washington, PA, USA) and the resin polymerized for 24 hr at 55°C in a sealed oven purged with nitrogen. Resulting blocks were removed from the capsules and stored in capped glass tubes in a desiccator containing silica gel. Samples were sectioned with a diamond knife

on a Leica Ultracut UCT ultramicrotome (Leica AG., Austria) and sections collected on either 300 mesh formvar plus carbon coated nickel grids or 200 mesh formvar plus carbon coated copper grids for immunocytochemical staining.

Immunocytochemical staining

Localization of zeins either in the matrix or within protein bodies was done by immunocytochemical staining of the thin sections by the technique modified from Batterman-Azcona *et al.* (1999b). α-Zein and γ-zein specific rabbit polyclonal antibodies were provided by Dr. Brian Larkins, University of Arizona. Both antisera were tested for specificity prior to the immunocytochemical procedure, using the western-blot protocol described by Lending *et al.* (1988). Two dilution levels (1:1000 and 1:4000) were tested for each zein-specific antiserum with samples of the same protein concentration loaded into the wells of the gels. For the immunocytochemical staining, grids were floated section-side down on 40ul drops of reagents on a sheet of Parafilm displaced inside large petri dishes. Reagents were kept at room temperature, except for antiserum and conjugates, which were stored at 4°C. Incubations were carried out at room temperature (22 ± 2°C). Grids were first floated in 20mM Tris buffer pH 7.4, containing 150mM NaCl, 1% (w/v) BSA, 0.3% (v/v) Tween 20, and 0.02% (w/v) sodium azide *(TBS-T-B)* for 15 min. Then they were floated in drops of the primary antibody diluted at 1:1000 in 20mM Tris buffer pH 7.4, containing 150mM NaCl , 1% (w/v) BSA *(TBS-B)* and incubated for 2 hr. Grids were washed in a sequence of three 200ul aliquots of 20mM Tris buffer pH 7.4, containing 150mM NaCl and 0.3% (v/v) Tween 20 *(TBS-T)* placed in spot plate wells, before being floated in TBS-TB for 5 min. Grids were blotted and

incubated for one hr in the colloidal gold/goat anti-rabbit solution diluted in TBS-T-B inside a petri dish covered with aluminum foil. After incubation, grids were blotted and washed in aliquots of TBS-T in spot plate wells. In sequence, grids were blotted and rinsed with distilled water and placed on a filter paper to dry. The double immunogold labeling for α and γ-zeins was performed running first a protocol for the α-zein antiserum and then, a subsequent protocol for the γ-zein antiserum. No excessive drying had taken place in the grids between runs. Two colloidal gold goat anti-rabbit conjugates of 12 nm and 18nm (Jackson Immunoresearch Laboratories, INC., West Groove, PA) were used in the labeling of α and γ-zeins, respectively. Dilution was 1:30 and 1:20 for the smallest and the largest particle colloidal gold conjugates, respectively. For each treatment there was one control grid that has been not incubated with the primary antibody during the imunnolabeling. Grids were not stained with either uranyl acetate or lead citrate in order to avoid masking the result of the cytochemistry. Sections were examined on either a Zeiss EM912 Omega transmission electron microscope (Leo Electromicroscope GmbH, Germany) with an accelerating voltage of 80kV, or a JEM 2000 EXII (JEOL USA In., Peabody, MA) transmission electron microscope at an accelerating voltage of 100kV.

Experimental design

The experiment was set as a factorial design with two levels for maize type (QPM and normal) and seven levels for specific mechanical energy (SME) (0, 55, 75, 160, 250, 300, and 430kJ/kg) to a total of 14 treatments with two replicates of extrusion (n=28). The level 0 corresponded to raw maize grits. Measurement of density of the zein bands was done in triplicate and values averaged. Data resulting from densitometry were

analyzed using ANOVA followed by LSD test for mean treatments comparison at $p<0.05$. The software used for data analysis was SYSTAT 8.0 (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Alpha and gamma-zein quantification

The SDS-PAGE patterns of zeins extracted from normal and QPM maize grits are presented in Figure 3.1.

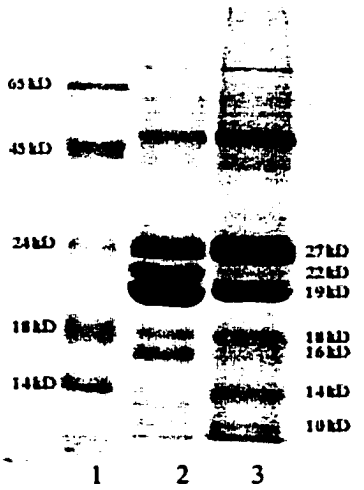


Figure 3.1. SDS-PAGE patterns of zeins extracted from normal and QPM maize grits following the procedure by Wallace *et al.* (1990). Lanes: 1) molecular weigh marker, 2) normal maize and, 3) QPM

Electrophorectic pattern for the normal maize sample revealed bands of the α-zeins of 22kD and 19kD, and also γ-zeins of 50 kD, 27kD, and, 16kD. In addition, a band corresponding to δ-zein of 18kD was detected for the normal maize but 14 kD β-zein and

the 10kD δ-zeins were absent. On the other hand, the 16kD γ-zein was absent for the QPM sample and the 22 kD α-zein and 10kD δ-zein were present only as faint bands. The 18kD δ-zein was also present in the QPM sample, as well as a higher amount of γ -zein. In general, α- and γ-zeins were found to be the most abundant fractions in the maize grits, despite the differences in band intensities between normal and QPM samples.

Quantitative analysis of the zein bands present in the SDS-PAGE gel using densitometry (Table 3.1.) demonstrated that α-Zein (19kD) was the predominant prolamin fraction in the normal maize grits, followed by the γ-zein (27kD). In contrast, γ-zein (27kD) was the predominant fraction in the QPM sample.

Table 3.1
Alpha and gamma zeins in normal and QPM maize grits determined by densitometry

Zein fraction	kD	Maize type	
		Normal	QPM
		Relative units *	
Alpha	22	316.5 ± 16.1 [a]	160.0 ± 8.6 [b]
	19	620.3 ± 0.32 [a]	349.6 ± 4.8 [b]
Gamma	27	499.8 ± 3.1 [b]	1010.5 ± 5.7 [a]
	16	169.9 ± 3.5 [a]	99.5 ± 1.8 [b]
	50	147.1 ± 2.4 [b]	280.6 ± 5.2 [a]

* Mean ± standard error
**Means followed by the same lower case letter superscript within a column are not significantly different at $p<0.05$, according to LSD test

In general, the content of α-zein (19 and 22kD) in the normal maize grits was near twice as high as in QPM ($p<0.05$). On the other hand, data for QPM grits indicated considerably elevated content of γ-zeins (M_r 50 kD, 27kD, and 16kD) ($p<0.05$) when compared to normal maize grits. Similar results were reported by Wallace *et al.* (1990) and Paiva *et al.* (1991), who compared composition of zeins in the endosperm of various maize genotypes. In the study carried out by Paiva and coworkers (1991), content of γ-zein (27kD), which was measured through immunoassay ELISA, was reported to be 2 to 2.5 times higher in the endosperm of QPM genotypes than in normal maize genotypes. Also, QPM contained very little of the 22kD α-zein and significantly less 19kD α-zein.

Scanning electron microscopy

In both normal (Fig. 3.2) and QPM (Fig. 3.3) maize grits, starch granules(SG) appear tightly packed and covered by an adherent protein matrix (PM), which forms a sheet encasing starch granules (Fig. 3.2A). This structure resembles the horny or vitreous endosperm of maize (Wilson 1987).

The maize cultivars used to prepare the maize grits evaluated in this study were semi-flint types; therefore, it would be expected that the majority of maize grits would be small parts of vitreous endosperm. Protein bodies could be only identified in the normal maize grits (Fig. 3.2A). Perhaps, the close packing of the starch granules in the QPM samples may have obstructed the view into the matrix itself and thus, prevented the observation of the protein bodies.

Figure 3.2. Scanning electron micrographs of maize grits produced with grains of the normal semi-flint Brazilian genotype (BRS 3143). Polygonal starch granules (SG) are interconnected by a continuous protein matrix (PM) that holds clusters of protein bodies (PB).
Bar = 10μm

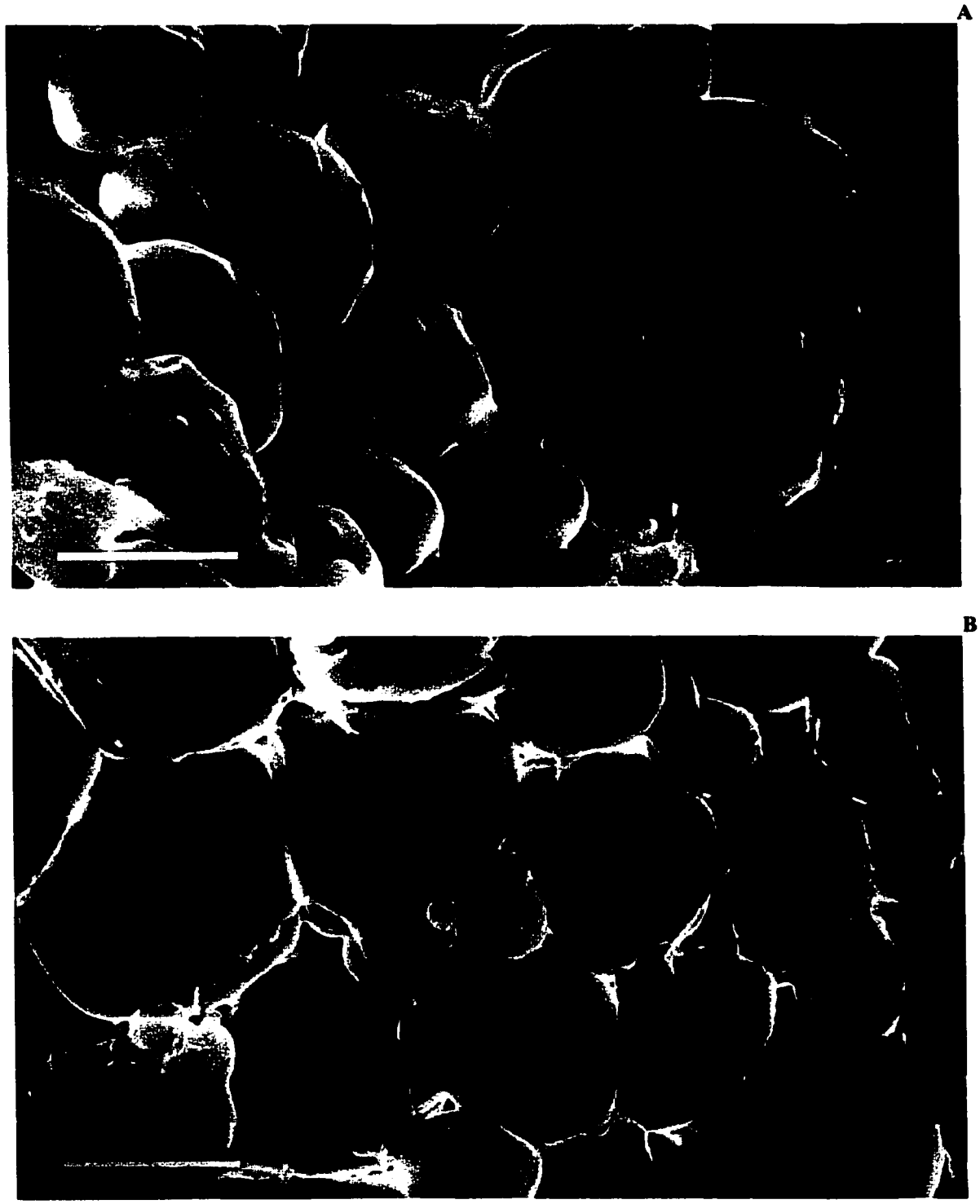


Figure 3.3. Scanning electron micrographs of maize grits produced with grains of the QPM semi-flint Brazilian genotype (BR 473). Starch granules (SG) are interconnected by a continuous protein matrix (PM).
Bar = 10μm

Researchers have reported that the endosperm of QPM grains contains smaller γ-zein rich protein bodies, averaging 1μm in diameter, which are condensed around the starch granules forming the protein matrix (Geetha *et al.* 1991; Larkins *et al.* 1995). On the other hand, protein bodies in the hard endosperm of normal maize average twice the size of those in QPM, and are predominantly constituted of α-zein (Lending *et al.* 1988). In the scanning micrographs of the normal and QPM maize grits used in this study, the differences in protein bodies could not be identified.

Transmission electron microscopy

Transmission electron micrographs of raw maize grits and extrudates obtained at various levels of specific mechanical energy are shown in Figures 3.4 through 3.14. α- and γ-zeins were immunolabeled with colloidal gold particles of 12 and 18nm, respectively. Bar size equals 1μm.

In raw normal maize grits (Fig 3.4) α and γ-zeins are distributed within the protein bodies in the protein matrix that surrounds the starch granules, and protein bodies have diameter of approximately 1-2μm. But contrary to the pattern described in the literature (Lending *et al* 1988; Lending and Larkins 1989) zeins are not only constrained within the protein bodies, rather they appeared spread in the protein matrix. γ- Zeins are also located on the surface and, though in minor quantities, within the starch granules, suggesting being associated with the starch granules (Fig 3.4B). The presence of γ-zein within the starch granule can also be observed in the images obtained of the raw QPM grits (Fig. 3.5A). For this sample, the protein matrix was also filled with α- and γ-zeins as can be seen by the immunogold localization of zeins, although protein bodies were not as clear

as for the normal maize grits (Fig.3.5B). The results of the SEM and TEM techniques support fewer protein proteins in the QPM grits.

The presence of proteins strongly associated at and within isolated starch granules of maize has been described by Mu-Foster and Wasserman (1998). The authors identified α-zeins of M_r19 and 22kD, β-zein of M_r10kD and γ-zein of M_r 27kD as major proteins located at or near the maize starch granule surface, whereas the starch branching enzyme IIb and the 60kD granule-bound starch synthase (GBSS), which is responsible for synthesis of amylose, were primarily located within the starch granules. Similar to the results described in the earlier study, Han and Hamaker (2002) have found proteins of M_r of approximately 25kD to 50kD, which correspond to the range of γ-zeins, in addition to the 60kD GBSS, to be granule-associated proteins in maize. Confocal electron microscopy after reaction of isolated starch with a protein-specific fluorescent dye has revealed that GBSS was located in internal concentric spheres within the granule structure and an unknown protein was located in the channels of maize starch. The findings of these earlier papers support the microstructures obtained for the maize grits. However, additional studies need to be done in order to investigate if this pattern is not an artifact due to smearing of the protein upon the sectioning of the specimens.

Extrusion at specific mechanical energy of 75kJ/kg promoted significant changes in the structure of starch granules and protein bodies for both QPM and normal maize grits (Fig. 3.6). Starch granules have lost their orderly structure, forming a melted matrix (Fig. 3.6B). Protein bodies were disrupted and fused together giving rise to protein aggregates containing both α and γ-zeins, which were surrounded by a melted starch network. However, the aggregation patterns significantly differed for QPM and normal

maize samples (Fig 3.7 A and B). Some intact protein bodies were still present in the QPM extrudate (Fig. 3.7B). The intact protein bodies appear to be filled mainly with γ-zein, although no morphometry was used to quantify the gold particles. This result confirms our hypothesis that the higher content of γ-zein in the QPM would confer resistance to shear to protein bodies within the extruder. Consequently, higher energy input would be necessary to break apart the γ-zein rich protein bodies present in the QPM grits. The images also allow for the observation that protein bodies in normal maize grits were disrupted at mechanical energy below 100kJ/kg, differently to the reports by (Batterman-Ascona *et al.* (1999a) when evaluating changes in protein bodies and α-zeins during extrusion of corn flour at different specific mechanical energy levels.

At an intermediate shear level (SME 160kJ/kg), protein aggregates became more evident in the extruded QPM sample, and the spherical shape of protein bodies, previously noted at specimens of extrudates obtained at SME 75 kJ/kg, was no longer present (Fig 3.8b). In both QPM and normal maize extruded samples, zeins were dispersed within the protein aggregated mass, but were not interacting to a large extent with the gelatinized starch (Fig. 3.8A and 3.8B). No difference in α- and γ-zeins dispersion pattern was noticed at this shear level when comparing images of the QPM and normal maize extrudates.

The trend observed for zein dispersion in samples extruded at a specific mechanical energy of 160kJ/kg is somehow dissimilar to that reported by Batterman-Ascona *et al.* (1999a) for corn flour extruded at similar shear level (SME 165 kJ/kg). The authors described disrupted protein bodies that have fused and formed elongated structures with α-zeins extensively dispersed through the system.

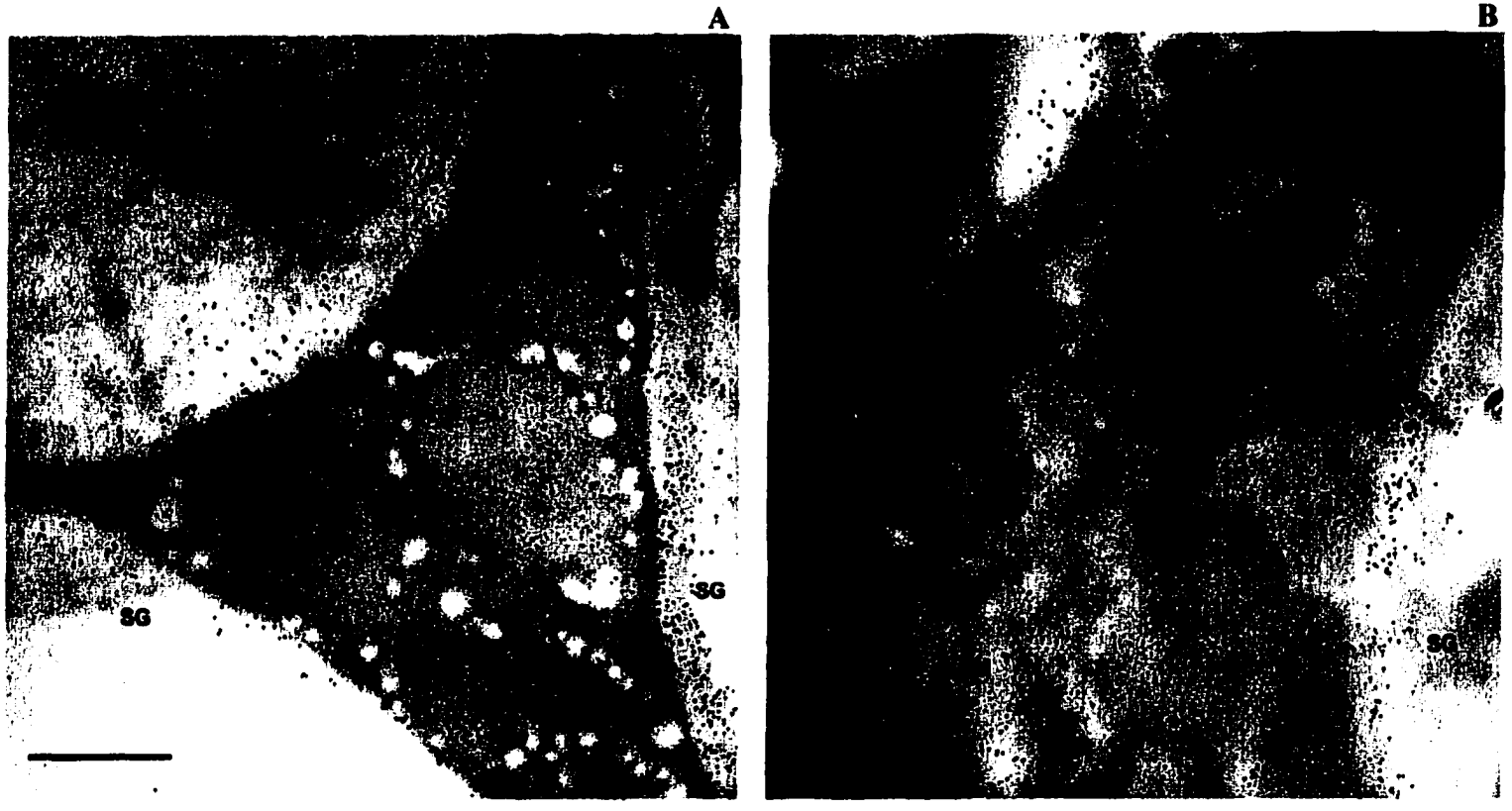


Figure 3.4. Transmission electron micrographs of normal raw corn grits showing protein bodies (PB) within the protein matrix (PM) surrounding starch granules (SG) (A). α- and γ-zeins were immunolabeled with colloidal gold particles of 12nm and 18nm diameter, respectively. Zeins fill most of the protein bodies, although γ-zein is also located on the surface of the starch granules and, in reduced quantities in its interior. Bar = 1μm

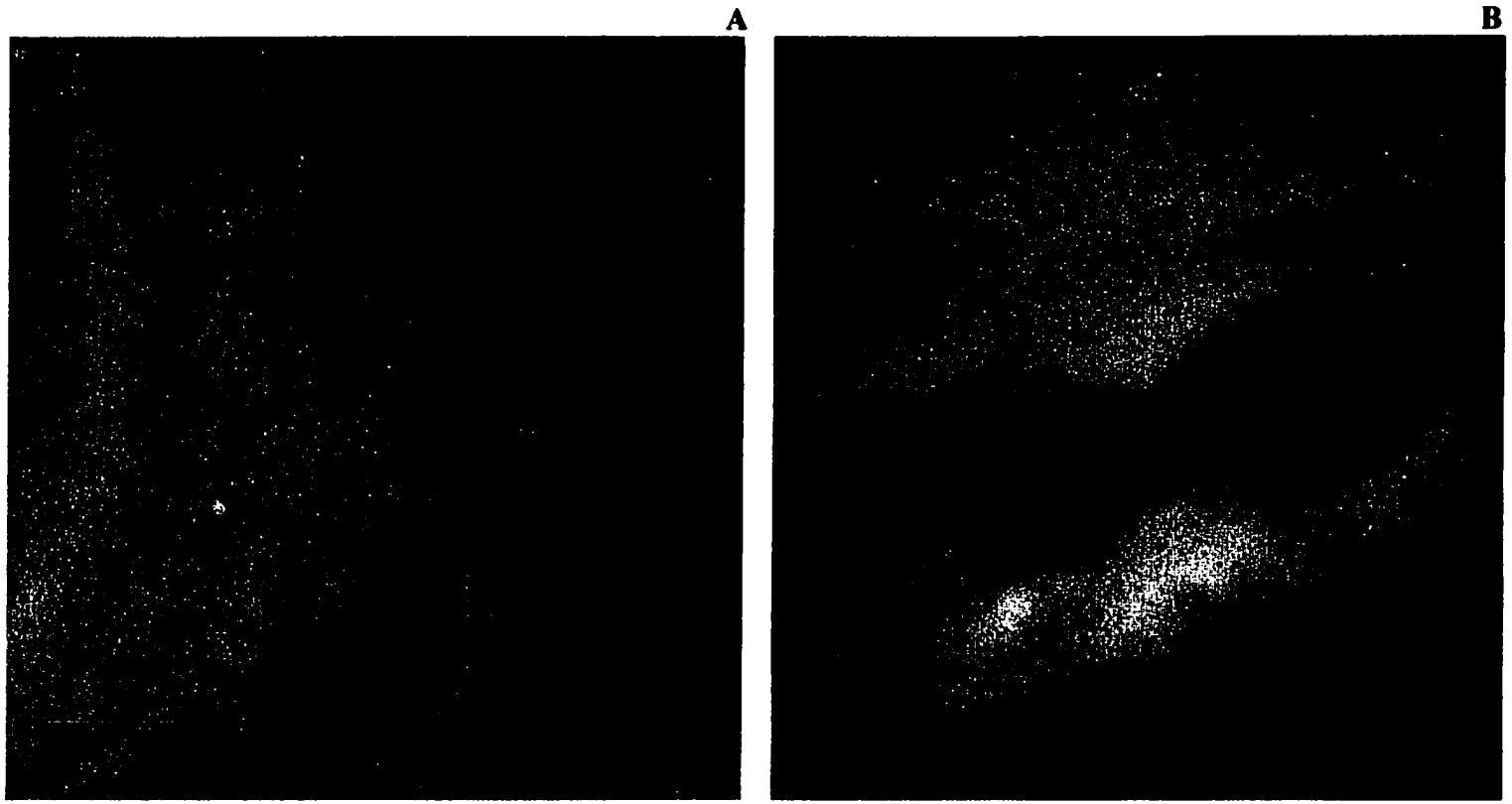


Figure 3.5. Transmission electron micrographs of raw QPM corn grits showing the protein matrix (PM) surrounding starch granules (SG)(A). Protein body (PB) is present within the protein matrix (PM) (B). α- and γ-zeins, which were immunolabeled with colloidal gold particles of 12nm and 18nm diameter, respectively, fill most of the matrix. Bar = 1μm

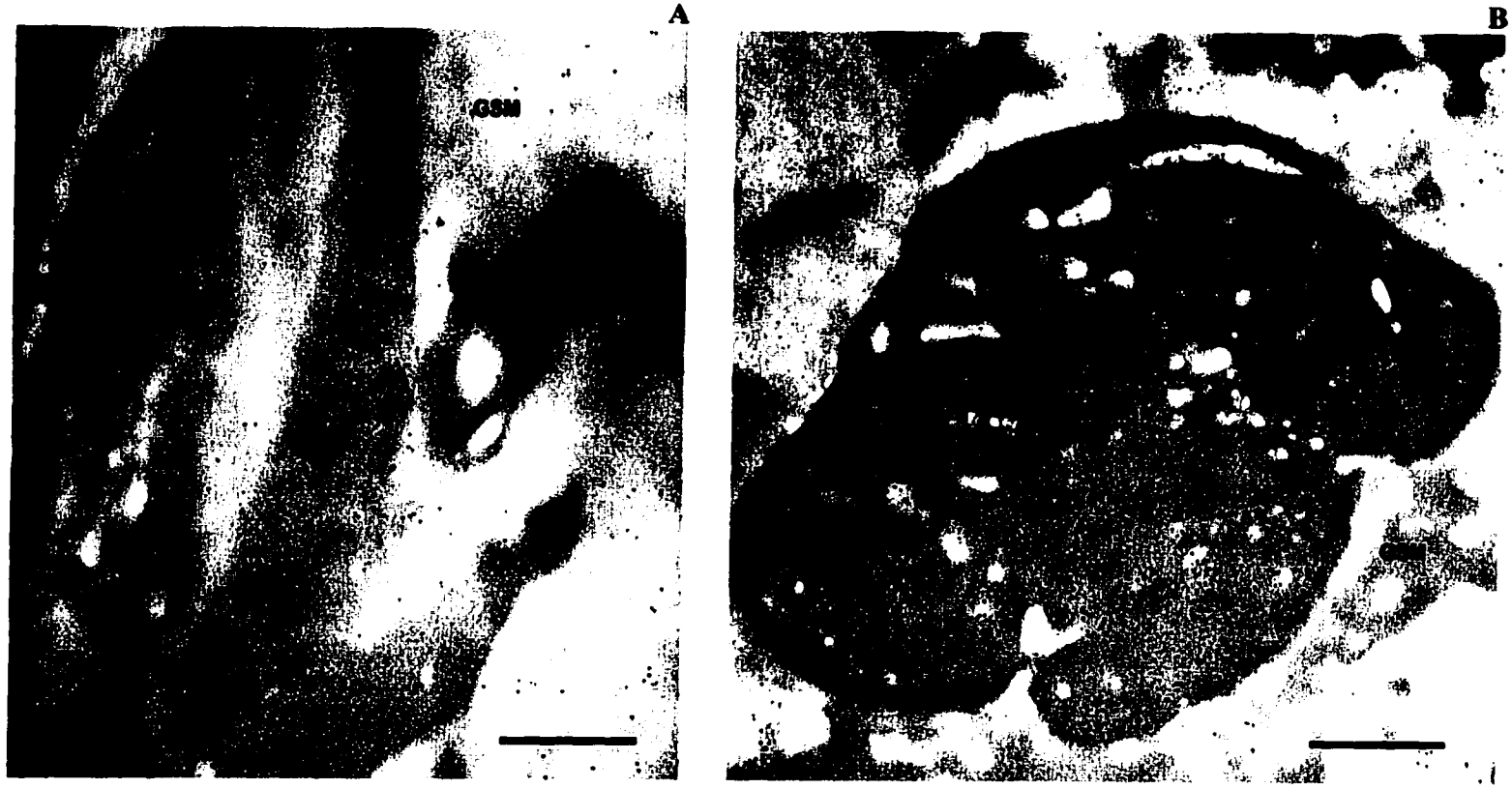


Figure 3.6. Transmission electron micrograph of normal (A) and QPM (B) corn grits extruded at specific mechanical energy of 75kJ/kg. Protein aggregates (PA) containing α- and γ-zeins are found surrounded by the melted matrix of gelatinized starch (GSM). α- and γ-zeins were immunolabeled with colloidal gold particles of 12nm and 18nm diameter, respectively . Bar = 1μm

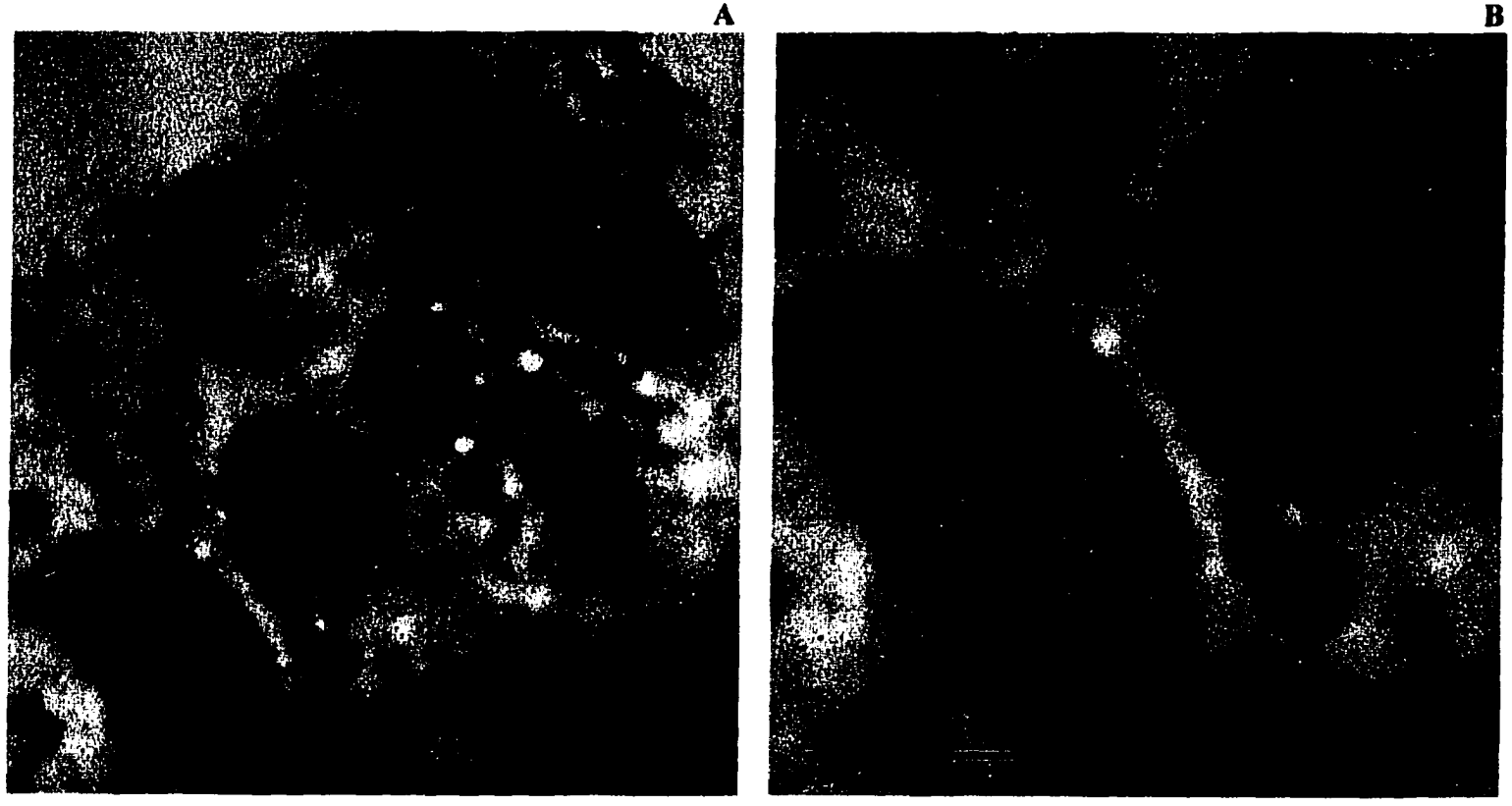


Figure 3.7. Transmission electron micrographs of QPM corn grits extruded at specific mechanical energy of 75kJ/kg. Some intact protein bodies (PB) were still present dispersed in the matrix of gelatinized starch (GSM). α- and γ-zeins were immunolabeled with colloidal gold particles of 12nm and 18nm diameter, respectively . Some intact protein bodies are still found in the gelatinized starch matrix. Bar = 1μm

Figure 3.8. Transmission electron micrographs of normal (A) and QPM (B) corn grits extruded at specific mechanical energy of 160kJ/kg with protein aggregates (PA) and the matrix of melted starch (GSM). α-zein and γ-zein were imunnolabeled with colloidal gold particles of 12nm and 18nm diameter, respectively. Bar = 1μm

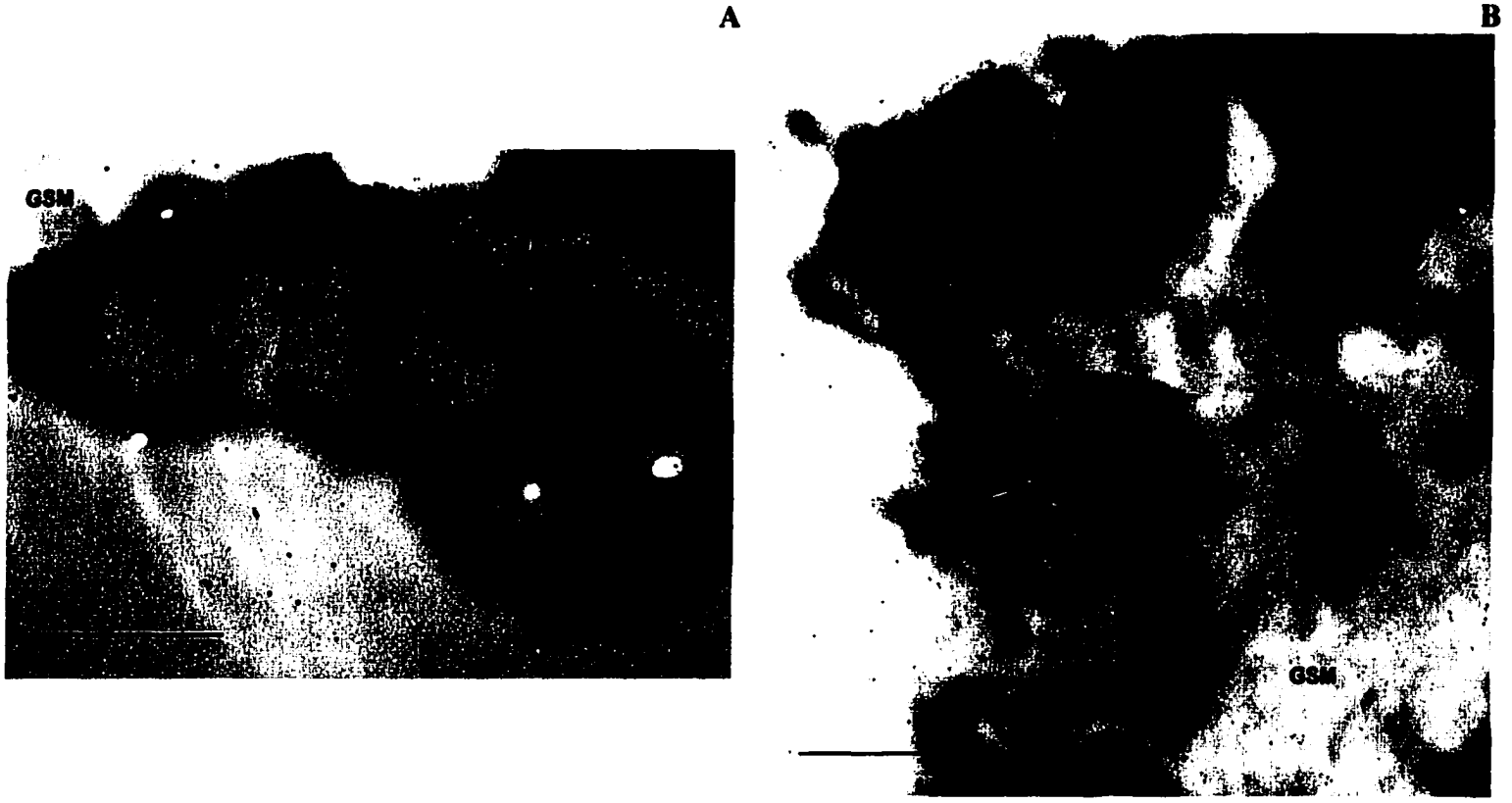


Figure 3.9. Transmission electron micrographs of normal (A) and QPM (B) corn grits extruded at specific mechanical energy of approximately 260kJ/kg showing protein aggregates (PA) and the matrix of gelatinized starch (GSM). α-zein and γ-zeins were imunnolabeled with colloidal gold particles of 12nm and 18nm diameter, respectively. Bar = 1μm

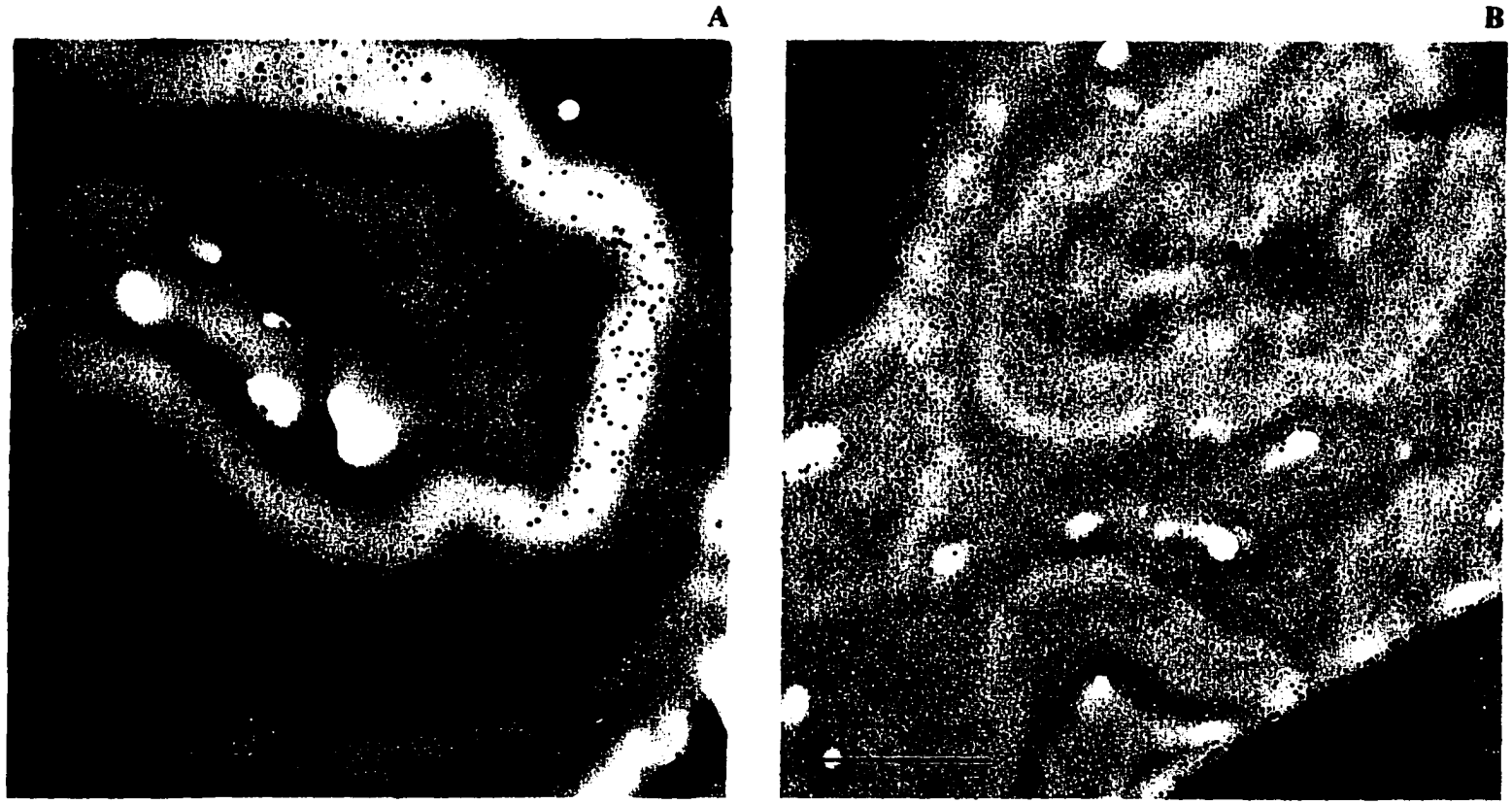


Figure 3.10. Transmission electron micrographs of normal (A) and QPM (B) corn grits extruded at specific mechanical energy of approximately 260kJ/kg. α-zein and γ-zeins were imunnolabeled with colloidal gold particles of 12nm and 18nm diameter, respectively. Bar = 1μm

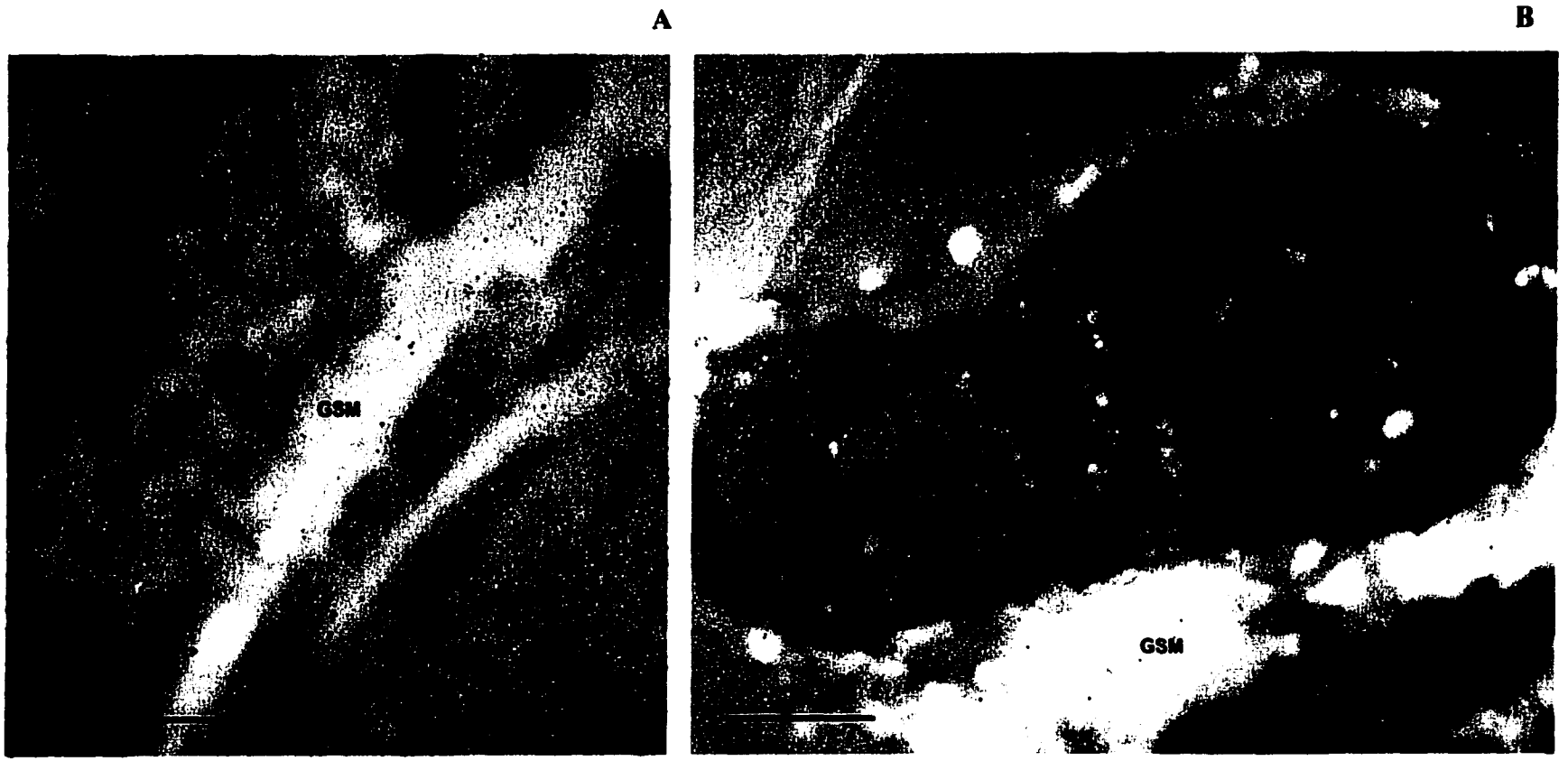


Figure 3.11. Transmission electron micrographs of normal (A) and QPM (B) corn grits extruded at specific mechanical energy of approximately 320kJ/kg. Protein aggregates (PA) appeared as large masses containing both α-zein and γ-zeins, which were imunnolabeled with colloidal gold particles of 12nm and 18nm diameter, respectively. The gelatinized starch matrix (GSM) can also be observed in the images. Bar = 1μm

Figure 3.12. Transmission electron micrographs of normal (A) and QPM (B) corn grits extruded at specific mechanical energy of approximately 320kJ/kg showing protein aggregates (PA) containning both α-zein and γ-zeins, which were imunnolabeled with colloidal gold particles of 12nm and 18nm diameter, respectively. Bar = 1μm

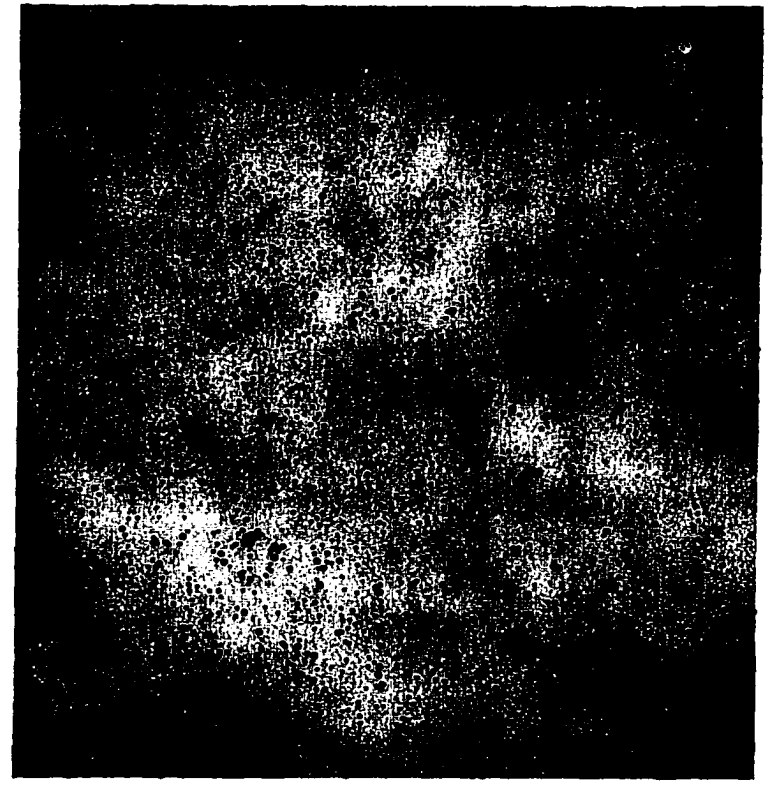


Figure 3.13. Transmission electron micrograph of normal corn grits extruded at specific mechanical energy of approximately 320kJ/kg showing zeins distributed within the protein matrix. α-zein and γ-zeins were imunnolabeled with colloidal gold particles of 12nm and 18nm diameter, respectively. Bar = 1μm

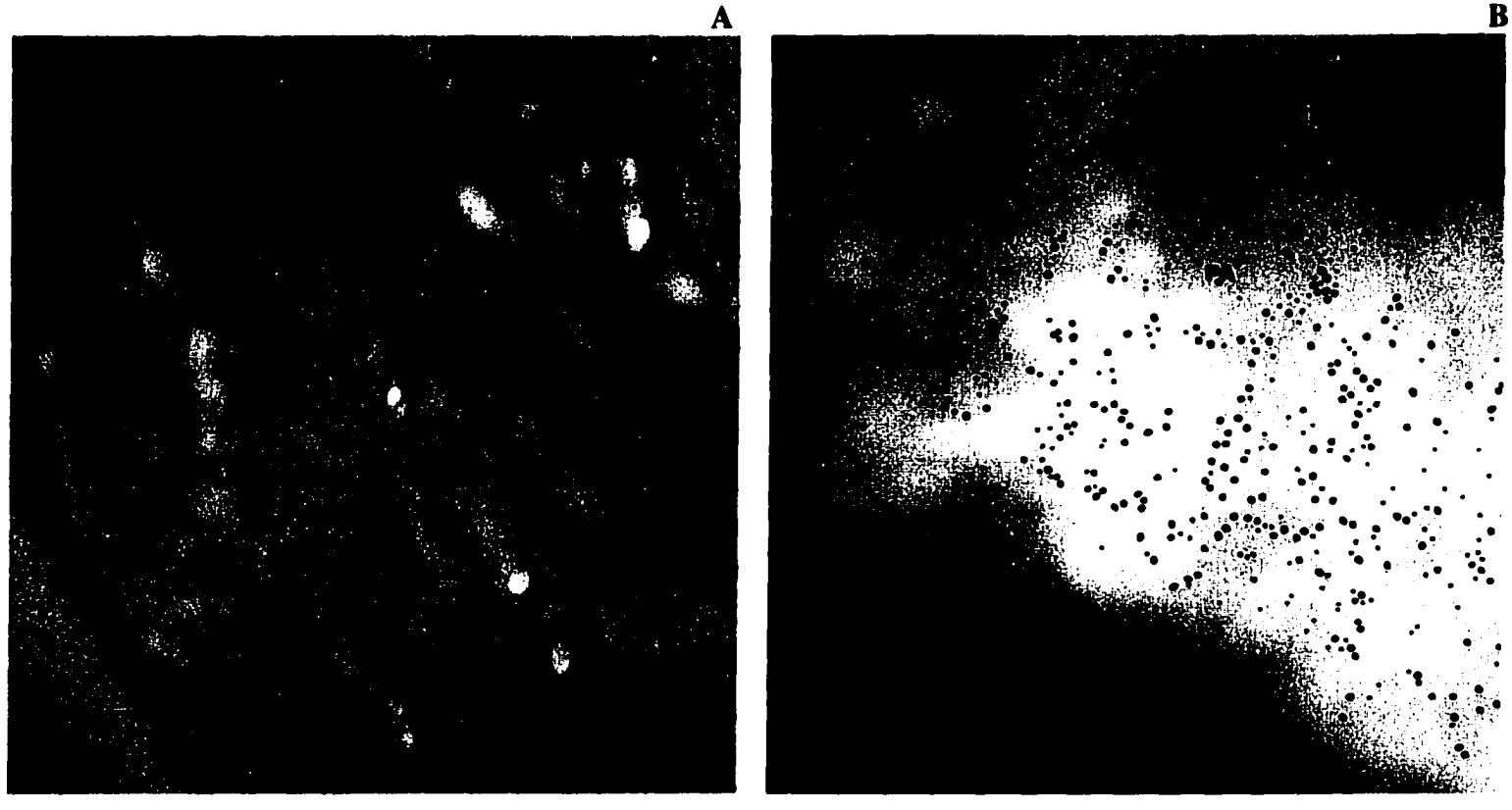


Figure 3.14. Transmission electron micrographs of normal (A) and QPM (B) corn grits extruded at specific mechanical energy of approximately 260kJ/kg. Protein aggregates contain α-zein and γ-zein, which were imunnolabeled with colloidal gold particles of 12nm and 18nm diameter, respectively. Bar = 1μm

An increase in the specific mechanical energy to 260kJ/kg did not seem to modify the aggregation pattern as compared to shear at 160kJ/kg (Fig 3.9). But, different patterns of protein aggregation were observed within specimens of the same samples (Fig 3.10). Tandjung (2000) described that extrusion of maize starch with added isolated zein at 10% level resulted in formation of aggregates of different sizes that were not evenly distributed but were surrounded by gelatinized starch. Similar to our findings, zeins were not found extensively dispersed through out the gelatinized matrix.

Harsher extrusion conditions (SME ~ 320kJ/kg) appeared to promote fusion of the preformed protein aggregates (Fig. 3.11) into larger and elongated protein masses, containing both α- and γ-zeins. Despite the fact that no morphometry was used to quantify the gold particles, QPM aggregates appeared to be predominantly composed of γ-zeins (Fig. 3.12B), which were primarily located at the periphery of the large aggregates. This structure was consistently found in the QPM grits extruded at this shear level. On the other hand, α-zeins seemed to fill most of the protein aggregate found in the normal maize extrudate obtained at SME of approximately 320kJ/kg (Fig. 3.12A and 3.13).

Extrusion of maize grits with an energy input of approximately 430kJ/Kg (Fig 3.14), which is usually observed in the industrial processing of cornflakes (extruded) and corn snacks (Moore 1999), resulted in formation of a continuous protein net, perhaps a result of zeins dispersion into large aggregated masses. For the QPM sample, γ-zein still appeared as the major protein within the net (Fig. 3.14B), whereas α-zein was the prevalent fraction in the normal maize extruded material (Fig.3.14A).

CONCLUSIONS

Maize grits of a QPM semi-flint cultivar contained higher content of γ-zein and reduced α-zein when compared to grits of a normal semi-flint maize cultivar. Extrusion caused significant changes in the microstructural organization of the three major components identified in the raw grits: starch granules, protein bodies, and the protein matrix. Starch granules have lost their orderly structure, gelatinized and melted to form a continuous matrix, whereas proteins aggregated into large masses diffused in the gelatinized starchy network. The high γ-zein content in QPM seems to confer resistance to disruption of protein bodies during extrusion. Therefore, high γ-zein rich protein bodies require higher energy input within the extruder for complete disruption than protein bodies found in normal maize. But, differences in dispersion of zeins in the system for the two maize types were not identified at high shear conditions evaluated in this study.

CHAPTER IV

COMPARISON OF CHEMICAL MODIFICATIONS IN MAIZE PROTEINS DURING EXTRUSION OF QPM AND NORMAL MAIZE GRITS AND THEIR IMPLICATION ON EXTRUDATE TEXTURE

ABSTRACT

The effect of high γ-zein content in Quality Protein Maize to chemical modifications of maize proteins during extrusion of maize grits and their implication on extrudate texture has been evaluated in this study. Proteins present in normal and QPM maize grits extruded at various levels of shear (SME 50, 75, 160, 250, 320, and 430 kJ/kg) were extracted using either urea, SDS, SDS+urea, and SDS+urea+2-ME containing buffers, and submitted to quantification through the BCA assay and separation through SDS-PAGE electrophoresis. Final extrudates were analyzed for breaking strength using the Warner-Braztler test. Chemical and textural analyses data were analyzed using ANOVA and the mean comparison LSD test was performed for significant effects at $p<0.05$. In general, proteins in QPM maize grits were less extractable in urea and SDS containing buffers than proteins in the normal maize grits ($p<0.05$). Addition of a reducing agent to the solvent significantly increased the protein extracted from both QPM

and normal maize unprocessed samples, although this effect was more evident for the QPM sample, indicating the importance of disulfide bonding to the structure of the protein in this sample. Extrusion promoted significant modifications in the chemistry of maize proteins, mainly zeins. After extrusion at the mid range of SME's, proteins became less soluble, perhaps due to protein aggregation. But harsh extrusion conditions (>320 kJ/kg) apparently favored the solubilization of zeins. In general, modifications in protein chemistry were driven by noncovalent and covalent interactions, although the disulfide bonding was apparently more important for the QPM samples. Chemical changes in proteins were identified as an important factor influencing brittleness of the final extrudates. Also, moisture negatively affected the brittleness of extrudates ($p<0.05$), regardless of maize type. QPM extrudates were more brittle than normal corn extrudates ($p<0.05$), perhaps due to the high γ-zein content in QPM. The use of QPM grits replacing normal grits in the production of extruded corn-based products may promote extended bowl-life of extruded corn flakes as well as enhance crispness of fully expanded snacks.

INTRODUCTION

Extrusion has been widely used for the processing of maize grits and flours in the production of breakfast products and savory snacks (Moore, 1994; Riaz 2000). In general, breakfast cereals and ready-to-eat extruded products are expected to have specific quality attributes (e.g. color, flavor, texture, and bowl-life), which in turn depend not only on extrusion processing variables but also on the composition of the ingredients

(Halek and Clark 1992; Serna-Saldivar *et al.* 2001). During extrusion processing, the combined effects of shear, high temperature, and pressure promote molecular transformations in the components of the ingredients, which includes physical and chemical changes in biopolymers such as proteins and carbohydrates (Wasserman *et al.* 1992; Li and Lee 1996a; Camire 2000; Sevatson and Huber 2000). Those modifications are known to greatly influence the physical properties of the final extruded product (Areas 1992; Rokey 2000). Transformations in starch due to extrusion have been extensively described as the major factor influencing texture of maize-based extrudates, since starch is the major component of maize grits and flours (Serna-Saldivar *et al.* 2001). However, the formation of zein fibers during extrusion of maize derivatives has recently been shown to be an important factor influencing textural parameters in corn-based extruded foods. Batterman-Azcona (1998) observed that addition of commercial zein to cornstarch before extrusion caused strengthening of the final extrudates. Similarly, Tandjung (2000) demonstrated that purified zein, when added to starch, modified texture of extrudates. Starch-zein extrudates had higher brittleness and stiffness than cornstarch extrudates, after being conditioned to specific moisture levels. The influence of zein composition to protein-protein interaction during extrusion processing is still not well understood.

Zeins, the alcohol soluble prolamins of maize, comprise more than 60% of the total protein of the kernel and 70% of the total endosperm protein (Hamaker *et al.* 1995). In the maize endosperm, they form the spherical organelles called protein bodies, which are found within the protein matrix surrounding the starch granules (Lending and Larkins 1989). Sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) analysis revealed that

zeins are a mixture of polypeptides, the most predominant of which have molecular masses of M_r 50 kD, 27kD, 22kD, 18kD, 19kD, 16kD, 14kD, and 10kD (Wallace 1990; Woo *et al.* 2001). But, based on their structures, rather than their differences in mobility or solubility, zeins were further classified in four groups. The complex group of the closely related polypeptides of M_r 22kD and M_r 19kD are called α-zein (Esen 1986). The M_r 14 kD protein is β-zein, whereas, the M_r 50kD, 27kD and 16 kD proteins are termed γ-zeins. The final polypeptides of M_r 18kD and 10kD are referred to as δ-zein (Lopes and Larkins 1992). Zein fractions are distinguishable in their amino acid sequencing, chain size, and spatial organization within the protein bodies (Esen 1986). α-Zein constitutes 75 to 85% of the total zein, and ranges in size from 210 to 245 amino acids (Lopes and Larkins 1992). These polypeptides have high contents of glutamine, leucine, alanine, and proline, which are predominantly hydrophobic amino acids (Shotwell and Larkins 1989). Contrary to α-zein, γ-zein accounts for 5-10% of the total zein in the endosperm of normal maize, and its 27kD polypeptide is a cysteine-rich (7%) fraction of 180 amino acids, containing also exceptionally high levels of proline (25%). The shortest polypeptide chain is the 10kD δ-zein, which is comprised of 130 amino acids, and contains significant amounts of proline, leucine, and the sulfur-containing amino acids, methionine and cysteine (Larkins and Lopes 1992). β- and γ-zein contain smaller amounts of leucine and other hydrophobic amino acids, and much higher amounts of cysteine than α-zein. On a mole percent basis, β-zein has about 5 mol% cysteine, and γ-zein has 7 mol%, whereas, α-zein contains only 1mol% cysteine (Esen *et al.* 1981; Paulis 1981, Lopes and Larkins 1992). Early in development, protein bodies in the endosperm of normal maize have both β- and γ-zeins, but little or no α-zeins. In the final stages of

protein body maturation, α-zeins fill most of the core of the protein body surrounded by a thin layer of β and γ-zeins (Dombrink-Kurtzman and Wilson 1992). Therefore, in the normal maize genotype, the predominant zein proteins are the 22 kD and 19kD α-zeins, followed by the 27kD γ-zein and the 14kD β-zein (Larkins *et al.* 1995). In contrast, the 27kD γ-zein is the most abundant zein fraction in Quality Protein Maize (QPM) genotypes (Paiva *et al.* 1991; Larkins and Lopes 1992; Moro *et al.* 1995).

Proteins undergo conformational and chemical modifications during extrusion such as denaturation and aggregation through covalent and noncovalent forces (Camire 1990; Wasserman *et al.* 1992; Li and Lee 1996a; Batterman-Ascona *et al.* 1999a). Thus, amino acid composition may play an important role in the modification of protein during extrusion. Considering that γ-zein contains less hydrophobic and more sulfurous amino acids when compared to α-zein, it could be hypothesized that the effect of extrusion on zein present in QPM maize grits is different than that of normal maize grits. The higher content of γ-zein in QPM may provide stability to protein bodies structure to the extruder shear, requiring higher specific mechanical energy to disrupt these structures during extrusion. But once disrupted, dispersion of zeins would occur to a greater extent and promote larger aggregation patterns through disulfide bonding. This in turn may promote increased hardness of corn-based extrudates. Considering this possibility, the objectives of this research were: a) to study the effect of extrusion on chemical modifications of zeins during processing of QPM and normal maize grits; b) to assess the influence of zein composition of QPM and normal maize grits in the chemical changes of maize prolamins during extrusion; c) to determine whether use of QPM leads to changes in texture of corn-based extrudates.

MATERIALS AND METHODS

Materials

Brazilian maize genotypes *QPM BR 473* and normal *BRS 3143* were grown at Embrapa- Maize and Sorghum Research Center in Sete Lagoas, Minas Gerais State, Brazil, during the 2001 growing season. Grains were dehulled and degermed with an industrial abrasive degerminator (Farsete, Sete Lagoas, MG, Brazil). Resulting flaking grits samples were milled in a dry milling plant (Moinho Jóia, Belo Horizonte, MG, Brazil) to coarse grits (800μm). Further grinding with an abrasive disc mill (Buhler-Miag, model MLI-204, Minneapolis, MN) provided smaller particle size grits (~550μm) that is typically used for the large-scale production of corn-based snacks. Grits were analyzed for moisture content according to the method 44-19 (AACC 1995), and a calculated volume of distilled water was added to each sample to final moisture of 22%. Samples and added water were mixed thoroughly for 20 min inside plastic bags for homogenization. Samples were allowed to equilibrate moisture for 24 hr at 10°C. To ensure final moisture content on samples, moisture was determined in the equilibrated samples following the method previously described. Conditioned samples were allowed to reach room temperature (~22°C) before being extruded.

Extrusion

Extrusion work was carried out at the facilities of the USDA/ARS/NCAUR, Peoria, IL, with the assistance of Dr. John Lawton. Each sample of maize grits was extruded in duplicate with a Brabender plasticorder single-screw extruder, PL2000 (Type

832500 C.W. Brabender Instruments, Inc., South Hackensack, NJ) with barrel diameter of 19mm, length to diameter ratio (L/D) of 30:1, and a die nozzle diameter of 3mm. Three screws with different compression ratios (1:1, 3:1, and dispersive) were combined to different screw speeds (20, 40, and 60 RPM) to provide different shear within the barrel, which resulted in six different levels of specific mechanical energy (SME) (50, 75, 160, 260, 320, and 430 kJ/Kg). Temperature was maintained constant with electrically heated, compressed air-cooled collars around the barrel in order to reach desirable levels of SME. Temperatures were 120, 160, 120, and 100°C, respectively, along the three zones of the extruder barrel (T1, T2, and T3) and the die opening (T4). Sample feed rate was determined during each individual run using the weight (g) of the extruded material collected for a period of 30 sec in the middle of the run. A die was used to identify the starting point of sample collection for feed rate determination and also to identify and separate samples of extrusion runs. The order of processing was randomly chosen and each extrusion run was brought to steady state as indicated by constant torque, before sampling and data collection. Torque (Nm) and screw speed (rpm) were recorded during processing on an interfaced computer for automatic data collection, with the Basic Program with Multiple Evaluation (version 4.0.2) (Brabender Instruments, Inc., South Hackensack, NJ). SME was calculated by dividing the net power input to the screw by the extrudate feed rate following the formula:

$$\text{SME (kJ/Kg)} = [\text{Torque (Nm)} \times \text{screw speed (rpm)} \times 2\pi] / \text{feed rate (Kg/hr)}$$

Resulting extruded rods were randomly collected in the air-cooling tunnel, and cut into approximately 20-cm length sample. Extrudates were air-dried at 40°C to final

moisture of approximately 9%, cooled at room temperature, and stored in sealed plastic bags at –20°C until texture analysis. A sample from each treatment was ground using a cyclone mill (Cyclotec 1093, Tecator, Hoganas, Sweden) with a 0.5mm screen before chemical analysis.

Protein and moisture determination

Unprocessed grits and extrudates were analyzed for protein and moisture contents according to the AACC approved methods 44-15 A and 46-13, respectively (AACC 1995). Protein content was calculated by multiplying Nx5.7

Protein extraction

Ground samples of unprocessed and extruded maize grits were further air-dried at 40°C to constant weight before protein extraction and stored in a desiccator at room temperature before analysis. Protein extraction was done with 30 mg samples using the procedure described by Batterman-Azcona (1998) with the following solvents: a) 6M urea buffered in 10mM Tris buffer (pH 8); b) 2% sodium dodecyl sulfate (SDS) buffered in 10mM Tris buffer (pH 8); c) 6M urea, 2% SDS buffered in 10mM Tris buffer (pH 8); d) 6M urea, 2%SDS, 2% 2-mercaptoethanol (2-ME) buffered in 10mM Tris buffer (pH 8). Sample to solvent ratio was 1:15 (w/v).

Protein extraction was done in triplicate with constant agitation for 3 hr at room temperature, followed by centrifugation in an Eppendorf centrifuge model 5415C (Brinkman Istruments Inc., Westbury, NY) at 14,000 rpm for 20 min. Aliquots of resulting supernatants were analyzed for protein content using the BCA (bicichoninic

acid assay) protein assay kit (Sigma Chemical Co., St. Louis, MO). Dilution of supernatants allowed compatibility of the urea and SDS concentrations present in the extraction buffers to the assay. Extracts containing 2-mercaptoethanol were treated with six volumes of cold acetone (-20°C) for protein precipitation and elimination of the interference of the 2-ME in the assay. Tubes containing extracts plus acetone were set on ice for 30 min. followed by centrifugation at 12,000 rpm for 15 min. Supernatants containing acetone and buffer were discarded and the resulting pellets were allowed to dry at room temperature for 20 min. Precipitated protein was brought to suspension with 2% SDS 10mM Tris buffer pH 8. For complete protein solubility, tubes were extensively vortexed and incubated at 37°C in a heat-controlled water bath for 30 min. Samples were allowed to reach room temperature before being analyzed with the BCA assay. Standard curves were run for each individual set of samples corresponded to a specific buffer. For this purpose, the standard protein, bovine serum albumin (Sigma Chemical Co., St. Louis, MO), was suspended in each of the extraction buffers.

SDS-PAGE of proteins extracted from maize grits and extrudates

Protein extracted from unprocessed and extruded samples were subjected to SDS-PAGE on a vertical mini gel system (Mini-PROTEAN II cell, Bio Rad, Hercules, CA) using a polyacrylamide 12.5% (w/v) separation gel and a 4% stacking (w/v) gel, following the method described by Laemmli *et al.* (1970). Extracted supernatants (75μl) were added of 25 μl sample solvent (3x concentrate) (6 % SDS, 0.3% bromophenol blue, 30% glycerol 0.1875M Tris at pH 6.8,) with and without a reducing agent (5% 2-ME) for reducing and non-reducing conditions. The mixture was homogenized in a vortex Genie 2

(Scientific Industries, Bohemia, NY) and heated in a boiling bath for 3 min. before being applied to the wells of the gel. An equal volume of sample was loaded into each well for similar maize type, regardless of the extraction scheme. Corrections were made in total volumes of samples loaded to the gel when QPM samples were used. This was due to the initial difference on protein content of normal and QPM maize grits. The Dalton marker VI (Sigma Chemical Co., St. Louis, MO) was used as a molecular marker. Electrophoresis was performed at 100 volts for 100 min. Gel staining was done with 0.025% Coomassie Blue R-250 in 50% ethanol and 10% acetic acid and destained in 10% acetic acid and 5% ethanol.

Texture analysis

Sample preparation

Prior to textural analysis, extruded maize flour samples were cut into 5 cm long rods and equilibrated to relative humidities of 32.5, 57.7, and 85%, or approximately equivalent to 8%, 12%, and 16% moistures at 25°C (Vertucci and Roos 1993). In order to achieve desirable moisture contents, extruded samples were left for approximately three weeks inside desiccators containing saturated solutions of either magnesium chloride, sodium bromide, or sodium chloride/potassium chloride, following the procedure described by Pollio *et al.* (1996). Desiccators were kept in a temperature-controlled room at 25°C ± 2°C. Equilibrium was checked by monitoring the weight of samples as suggested by Tandjung (2000). The actual moisture content of the equilibrated samples was determined after oven drying at 105°C for 24 hr, and reported as weight loss (Piazza and Mali 1997).

Warner-Braztler test

Brittleness of the QPM and normal flour extrudates was determined using a TA.HDi Texture analyzer (Stable Micro systems, Godalming, England). Dry strands of extruded samples 5 cm long were placed over a support of the Warner-Braztler (WB) blade one at a time and the maximum force to break each piece was recorded using a 50Kg load cell that was interfaced with a computer. Data were recorded as peak-force (g). The pre-test, test, and post-test speeds were 5, 5, and 4 mm/sec, respectively. The WB shear blade was programmed to penetrate the sample to a distance approximately 60% of the sample height with a force of 100-1,000 kg. The trigger was set in automatic mode for start when the registered force reached 15g. Breaking strength (g/mm^2) was calculated as the peak force, expressed in grams, divided by the cross sectional area of the extrudate. To calculate the cross sectional area, the diameter of each strand was determined prior to the test using a digital caliper model CD-6 (Mitutoyo Inc., Japan). Measurements were done on five replicate samples.

Experimental design and statistical analyses

The experiment was set as a factorial design with two levels for maize type (QPM and normal) and seven levels for specific mechanical energy (SME) (0, 55, 75, 160, 260, 320 and 430kJ/kg) for a total of 14 treatments with two replicates for extrusion (n=28). The level 0 of SME corresponded to unprocessed maize grits and was not included in the texture analysis study. Data were analyzed using ANOVA followed by either LSD or Tukey tests for mean treatments comparison at $p<0.05$. The software used for data analysis was SYSTAT 8.0 (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Protein and moisture contents

Results of protein and moisture contents of dried ground extruded samples are presented in Table 4.1. Percent moisture was not the same among extruded samples processed under different SME, even after air-drying at 40°C for 24 hr ($p<0.05$). Since screw design and screw speed were varied from the lowest to the highest shear levels, exposure to heat within the extruder was possibly altered. Therefore, moisture loss was also affected, rendering extrudates with various moisture contents in a range of 8.4 to 10.4%. Based on the results of moisture analysis, ground extruded samples, which would be used for evaluation through solubility test and SDS-PAGE, were allowed to dry in a forced-air oven at 40°C to constant weight and were stored in sealed glass containers in a desiccator until being used for analyses.

Since percentage protein (dry weight basis) in extruded QPM samples was lower than for their extruded normal maize counterparts ($p<0.05$), results for the total protein soluble test (BCA) were normalized for protein content of the starting material to allow for comparison of solubility results. Also, amounts of QPM samples assayed through the SDS-PAGE were adjusted to ensure that the same total protein would be loaded for normal and QPM samples. This correction provided fair comparisons of molecular weight modification of proteins in normal and QPM extruded samples.

Table 4.1.
Percent moisture and protein of unprocessed and extruded maize grits

Shear level	% Moisture		% Protein*	
(KJ/kg)	Normal	QPM	Normal	QPM
SME= 50	10.4 [a]	9.5 [a]	6.1 [c A]	5.9 [ab A]
SME = 75	8.4 [bc]	9.2 [bc]	6.6 [bc A]	5.7 [b B]
SME = 160	9.8 [a]	9.7 [a]	6.9 [b A]	5.7 [b B]
SME = 260	9.6 [a]	10.2 [a]	6.9 [b A]	5.9 [ab B]
SME = 320	10.1 [a]	10.3 [a]	7.2 [a A]	6.3 [a B]
SME = 430	9.3 [b]	9.1 [b]	7.5 [a A]	6.1 [ab B]
SEM	0.252		0.048	

* Dry weight basis, based on Nx5.7 conversion factor
** Means followed by the same lower case letter within a column are not significantly different at $p<0.05$, according to LSD test
*** Means followed by the same capital letter within a row are not significantly different at $p<0.05$, according to LSD test

Protein solubility

Quantitative data of protein solubilized in different extracting buffers are presented in Table 4.2. Extracting buffers were chosen to provide an understanding of the chemical modifications in proteins occurring during the extrusion of maize grits. Urea would disrupt hydrogen bonds, while SDS and 2-ME would disrupt hydrophobic interactions and disulfide bonds, respectively.

Differences ($p<0.05$) were observed in total protein extracted for the normal and QPM samples among the four buffers used in this study (Table 4.2). Also, extrusion processing was shown to be an important factor influencing solubility of maize proteins.

Proteins in unprocessed QPM sample were significantly more soluble in urea alone (3.37 mg/ml) than SDS alone (1.43 mg/ml), indicating the importance of hydrogen bonding over hydrophobic interaction in their structure (Fig. 4.1).

Table 4.2
Protein (mg/ml) extracted from unprocessed and extruded maize grits using different extracting buffers and quantified through the Bicichoninic Acid Assay (BCA)

Processing levels	Maize type: Normal — Extracting buffers: Urea	SDS	Urea + SDS	Urea + SDS + 2-ME	Maize type: QPM — Extracting buffers: Urea	SDS	Urea + SDS	Urea + SDS + 2-ME
	Protein (mg/ml)							
Unprocessed grits	3.70 $^{Ca\alpha}$	2.68 $^{Ca\alpha}$	6.32 $^{Ba\alpha}$	9.39 $^{Aa\alpha}$	3.37 $^{Ba\alpha}$	1.43 $^{Ca\alpha}$	3.52 $^{Ba\beta}$	7.07 $^{Ad\beta}$
SME= 50	2.63 $^{BCab\alpha}$	1.52 $^{Ca\alpha}$	3.85 $^{Bb\alpha}$	6.49 $^{Ab\beta}$	1.54 $^{Bab\alpha}$	1.03 $^{Ba\alpha}$	1.86 $^{Ba\beta}$	10.71 $^{Aab\alpha}$
SME= 75	1.99 $^{Cab\alpha}$	2.03 $^{Ca\alpha}$	4.21 $^{Bb\alpha}$	6.36 $^{Ab\beta}$	1.96 $^{Bab\alpha}$	1.38 $^{Ba\alpha}$	2.36 $^{Ba\beta}$	9.86 $^{Abc\alpha}$
SME=160	1.60 $^{Cb\alpha}$	1.55 $^{Ca\alpha}$	3.60 $^{Bb\alpha}$	6.40 $^{Ab\beta}$	1.41 $^{Bab\alpha}$	1.09 $^{Ba\alpha}$	1.93 $^{Ba\beta}$	8.38 $^{Acd\alpha}$
SME=260	2.49 $^{Bab\alpha}$	1.99 $^{Ba\alpha}$	3.34 ^{Bb}	9.11 $^{Aa\alpha}$	1.28 $^{Bb\alpha}$	1.35 $^{Ba\alpha}$	2.57 $^{Ba\alpha}$	8.52 $^{Acd\alpha}$
SME=320	1.94 $^{Cab\alpha}$	2.42 $^{Ca\alpha}$	4.60 $^{Bab\alpha}$	9.43 $^{Aa\beta}$	1.70 $^{Bab\alpha}$	1.31 $^{Ba\alpha}$	2.20 $^{Ba\beta}$	12.06 $^{Aa\alpha}$
SME=430	2.41 $^{Cab\alpha}$	2.21 $^{Ca\alpha}$	4.46 $^{Bab\alpha}$	9.76 $^{Aa\alpha}$	2.23 $^{Bab\alpha}$	1.21 $^{Ba\alpha}$	2.48 $^{Ba\beta}$	9.47 $^{Abc\alpha}$
	SEM = ± 0.444							

Each value represents a mean of six values.
Differences between solvent (row) for each SME level of a same maize type are indicated by capital letters
Differences between levels of SME (column) for each solvent of a same maize type are indicated by lower case letters
Differences between means of total protein extracted with a specific buffer by maize type within same processing level are indicated by Greek letters.
Means of same maize type within the same row or column followed by the same letter are not significantly different at $p<0.05$, according to Tukey's test.

Twice as much protein was extracted with SDS from unprocessed normal maize sample (2.68mg/ml) than from QPM sample (1.43mg/ml).

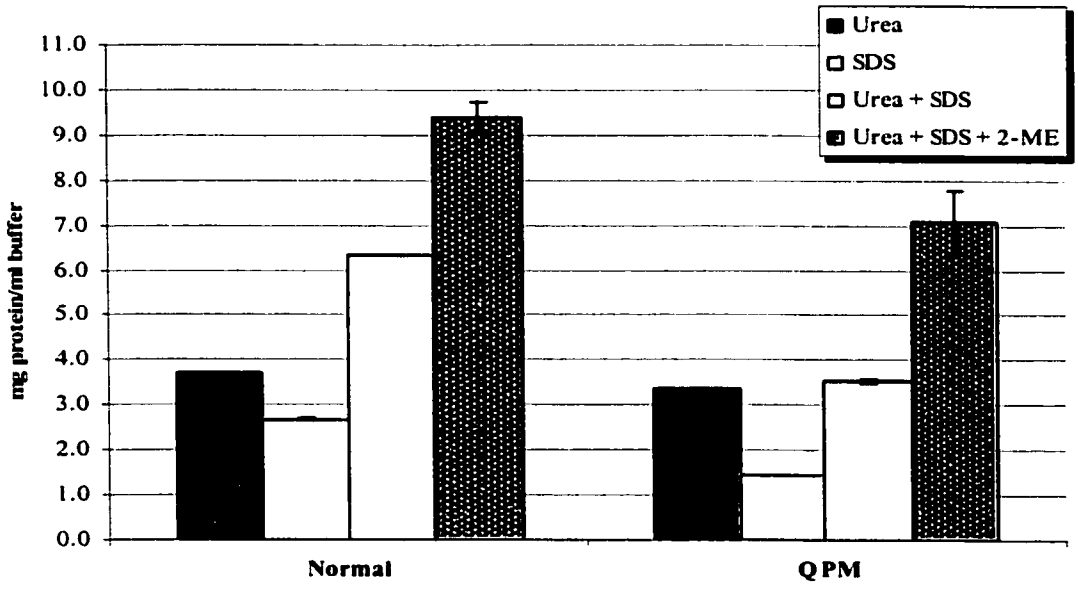


Figure 4.1. Total protein (mg/ml) extracted from normal and QPM maize grits with different extracting buffers as determined by the BCA method using bovine serum albumin (BSA) as standard.

Batterman-Azcona *et al.* (1999a) has described an opposite response on solvent solubility of protein in commercial corn flour extracted with either 6M urea or 2% SDS. It was found that urea alone extracted considerable less protein (~250μg/ml) than SDS alone (~750μg/ml).

In general, either urea or SDS alone extracted less protein from both unprocessed and extruded samples of normal maize than urea+SDS (Table 4.2). Conversely, the combination of urea and SDS did not provide the same synergistic effect on solubilizing proteins in extruded QPM samples, since there was no increase in protein extracted from those samples with urea+SDS, regardless of shear level. Hydrogen bond and hydrophobic

interactions apparently were not as important in stabilizing structure of maize proteins in QPM samples during the extrusion as they were for normal maize samples.

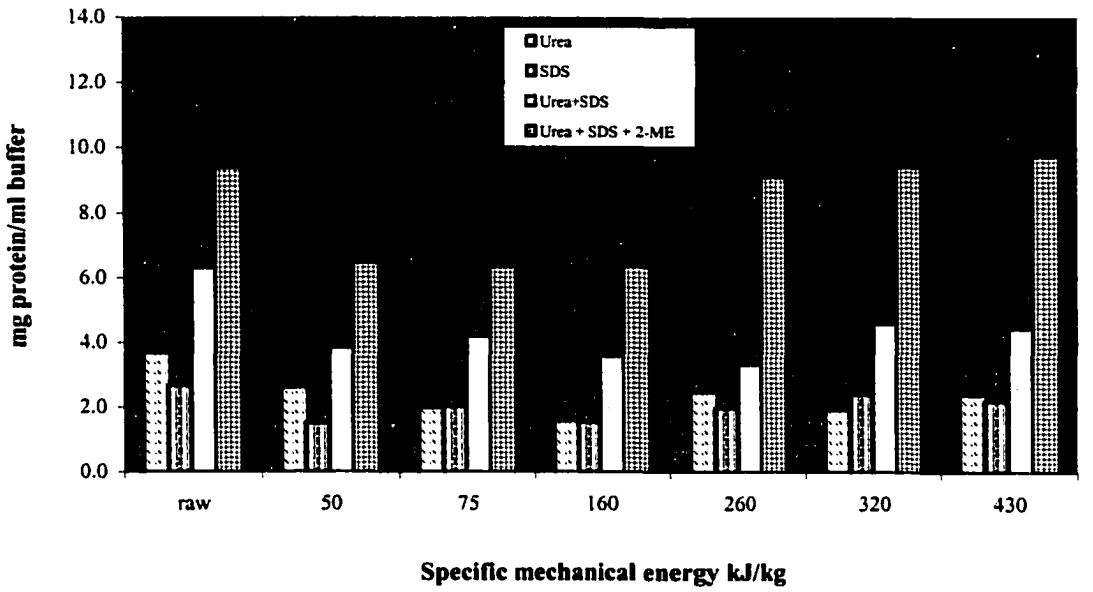
The addition of a reducing agent (2% 2-ME) to the urea+SDS buffer had a positive effect ($p<0.05$) on protein solubility from both unprocessed and extruded samples, regardless of maize type. This result provided evidence that proteins are highly covalent bonded after extrusion. It also demonstrated that disulfide bonding was the major force governing protein modification during extrusion processing independent of maize source. Similar to these findings, Batterman-Ascona *et al.* (1999a) reported that disulfide-bound protein complexes were prevalent in both unprocessed and extruded corn flour samples when studying the chemical modifications of zeins upon extrusion at various shear levels.

Despite the fact that 2-ME ($p<0.05$) increased the solubility of the maize protein, significantly less protein was taken into solution from QPM grits with addition of the reducing agent to the extracting buffer. This suggests that γ-zein rich protein bodies found in the unprocessed QPM samples were not as accessible to the solvent as the α-zein rich counterpart of the normal maize. It is interesting to note, however, that extrusion indeed affected solubility of maize proteins, since this trend was the opposite for the extruded samples at SME levels 50, 75, 160, and 320, from which considerably more protein was extracted with presence of a reducing agent from QPM samples than from the normal maize samples produced at the same SME levels. Considering the results of zein composition of maize grits presented in the previous chapter showing that the majority of the protein present in the QPM grits was γ-zein, it is possible that thermally-induced disulfide-bonding essentially drives modification of zeins due to extrusion. Based on a

model for modification of wheat proteins during extrusion by Li and Lee (1996), it could be speculated that low shear extrusion provided unfolding of the protein with exposition of S-S bonds within the γ-zein, which was not available in the unprocessed material, thus enhancing solubilization. Conversely, high shear (SME 250) could have favored inter- and intramolecular S-S exchange with protein aggregation, making the reducing agent less effective in breaking those bonds. At the highest level of shear (SME 430), dispersion of zeins and intramolecular S-S exchange through covalent bonding would be expected to happen to a greater extent, therefore, resulting in greater extractability.

Contrary to the results for QPM, extrusion below SME 260 had a tremendous negative impact on the solubility of protein of normal maize grits not only with the buffer containing a reducing agent, but also with the urea+SDS buffer (Fig. 4.2). This result is consistent with those from previous studies demonstrating reduction of protein solubility due to extrusion. Camire *et al.* (1991) observed a decrease in protein solubility after extrusion of corn meal at low shear conditions and identified protein aggregation as the major modification influencing protein solubility. These authors also reported that both noncovalent and covalent forces were responsible for protein aggregation upon extrusion of corn meal. Also, Li and Lee (1996) indicated that solubility of wheat proteins decreased significantly after extrusion in all solvents tested (0.01 M sodium hydroxide, 0.5M sodium chloride, 70% ethanol, 0.1 M hydrochloric acid, 0.05 M sodium phosphate buffer pH 7 and 8, 6M urea, 1% SDS, and 2% 2-ME) except for an aqueous solvent containing both SDS and 2-mercaptoethanol, which suggested that nonspecific hydrophobic interaction and intermolecular disulfide bonding played a major role in the

A



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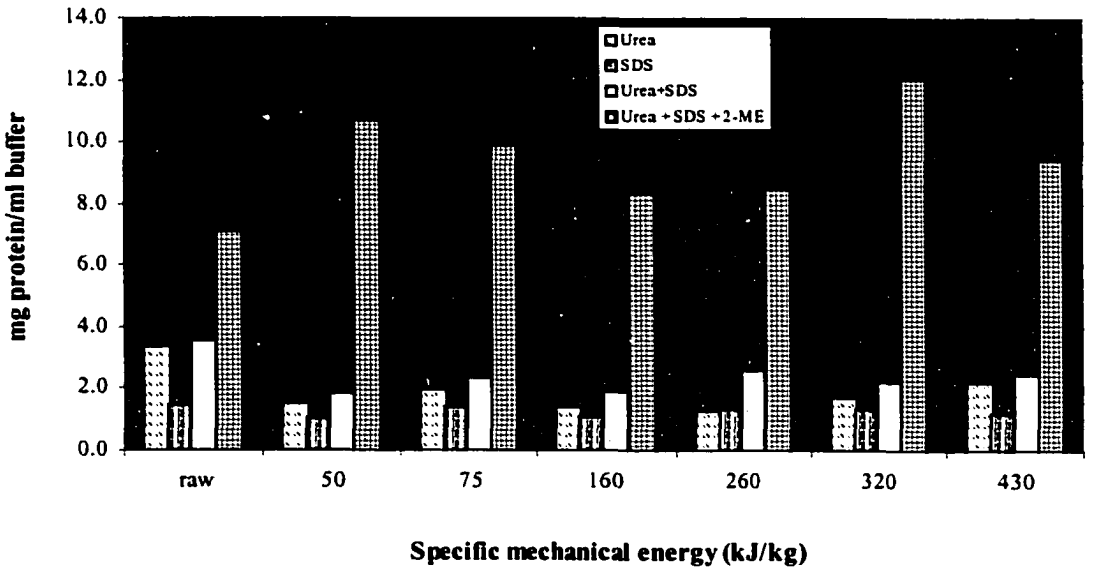

Figure 4.2. Total protein (mg/ml) extracted from unprocessed and extruded normal (A) and QPM maize grits (B) applying different extracting buffers as determined by the bicichoninic acid (BCA) method using bovine serum albumin (BSA) as standard.

chemical modifications of wheat proteins during the extrusion. SDS-PAGE analysis provided further evidence for protein aggregation, which was related to an increase in molecular weight of glutenins and gliadins, resulting in a decrease of protein solubility. Previous researchers also reported similar results for corn meal (Racicot *et al.* 1981) and wheat (Ummadi *et al.* 1995).

SDS-PAGE

This procedure provided separation and identification of soluble protein fractions and chemical modifications of zeins during extrusion processing. The molecular weight (MW) distribution profiles obtained from SDS-PAGE under reduced and unreduced conditions are presented in Figures 4.3 through 4.6.

Electrophoretic patterns for QPM and normal maize samples were different. Also, banding patterns were very distinct among samples of the same maize type when analyzed under reducing and non-reducing conditions. Moreover, differences in the intensity of the bands in the gels confirm the results of the BCA test, showing that protein in both unprocessed and extruded samples was less extractable in urea and SDS alone or combined over urea+SDS+2-ME (Fig. 4.3 through 4.5). SDS-PAGE gels additionally confirmed that proteins, mainly those with high molecular weights, were extracted to a lesser extent from unprocessed and extruded QPM samples than from normal maize by SDS alone or combined to urea.

Significant differences were observed also in the number and position of bands at the high molecular weight (HMW) region of gels of the extruded normal maize samples extracted with either SDS or urea upon electrophoresis under unreduced conditions.

Nevertheless, they did not differ from the ones present in unprocessed grits. But the high molecular weight bands disappeared when normal maize and QPM extruded samples were submitted to reduced SDS-PAGE, giving rise to more intense bands at the intermediate molecular weight region (20 to 27 kD), which correspond mainly to the α-zeins. It appears that larger MW peptides seen in the electrophoretic pattern under non-reducing conditions were stabilized by disulfide bonding. The result also indicates that high molecular weight bands in normal maize samples were extractable in urea (Fig. 4.3A) and somewhat in SDS (Fig. 4.4A) but not those in the QPM samples (Fig 4.3C and 4.4C). Those bands are possibly dimers of 22kD and 19kD α-zein and, 27kD γ-zein since these bands seem to migrate to the typical position of these three zein fractions upon reducing (Fig. 4.3B and 4.4B). Perhaps, α-zein in QPM is not present in the dimer form.

In addition to the initial observations, polypeptide banding of α-zeins (19 and 22 kD) and the polymeric form of the 22kD polypeptide (44kD) were similar for the unprocessed and extruded normal maize samples extracted with either urea or SDS alone or combined (Fig. 4.3 through 4.6) upon electrophoresis under non-reducing conditions. Conversely, the 19kD α-zein was the most predominant fraction extracted from both unprocessed and extruded QPM samples. An absence of the 27kD γ-zein band was observed in the gels for the extruded QPM samples and, both 27kD and 16kD bands for the normal maize extrudates, even under reducing conditions. Perhaps those proteins polymerized to higher molecular weigh polypeptides and were not able to enter the running gel.

The addition of a reducing agent (2-ME) to the extracting buffer not only considerably increased the amount of protein extracted from unprocessed and extruded

maize samples but reduced the differences in total protein extracted of the normal and QPM samples (Figure 4.6). Those bands were more intense for all levels of extrusion, regardless of maize type. Band of the methionine/cysteine-rich polypeptide δ-zein (14kD), could be detected for the QPM extrudates extracted in presence of 2-ME (Fig.4.6 B), which provides evidence for zein polymerization through disulfide bonding upon extrusion of QPM grits. The γ-zein (27kD), a cysteine-rich polypeptide, was only present in extruded normal maize samples produced at the shear levels above SME 160kJ/kg, and indicates that disulfide bonding played a major role in the maize protein aggregation upon extrusion at intermediate shear levels.

The very unique protein banding pattern for the intermediate and low molecular weight regions for samples extruded at lower shear (SME= 50 through 160) may be explained by the formation of protein aggregates, which might have impaired protein extractability (Fig. 4.6). Additional proof of polymerization is the fact that the band of MW approximately 45kD remained with similar intensity at the highest shear levels for the normal maize extruded samples, despite the presence of a triplet at the 27 to 19kD positions. At levels of shear equal to or above 250 kJ/kg, larger aggregates that were possibly stabilized by disulfide bonding in the low SME levels, may have undergone depolymerization, and formed the more intense bands of γ-zeins and β-zeins seen in the lanes corresponding to the normal maize extrudates (Fig. 4.6 A). Those changes may have also occurred to the QPM samples, regardless of the absence of the 22kD α-zein polypeptide.

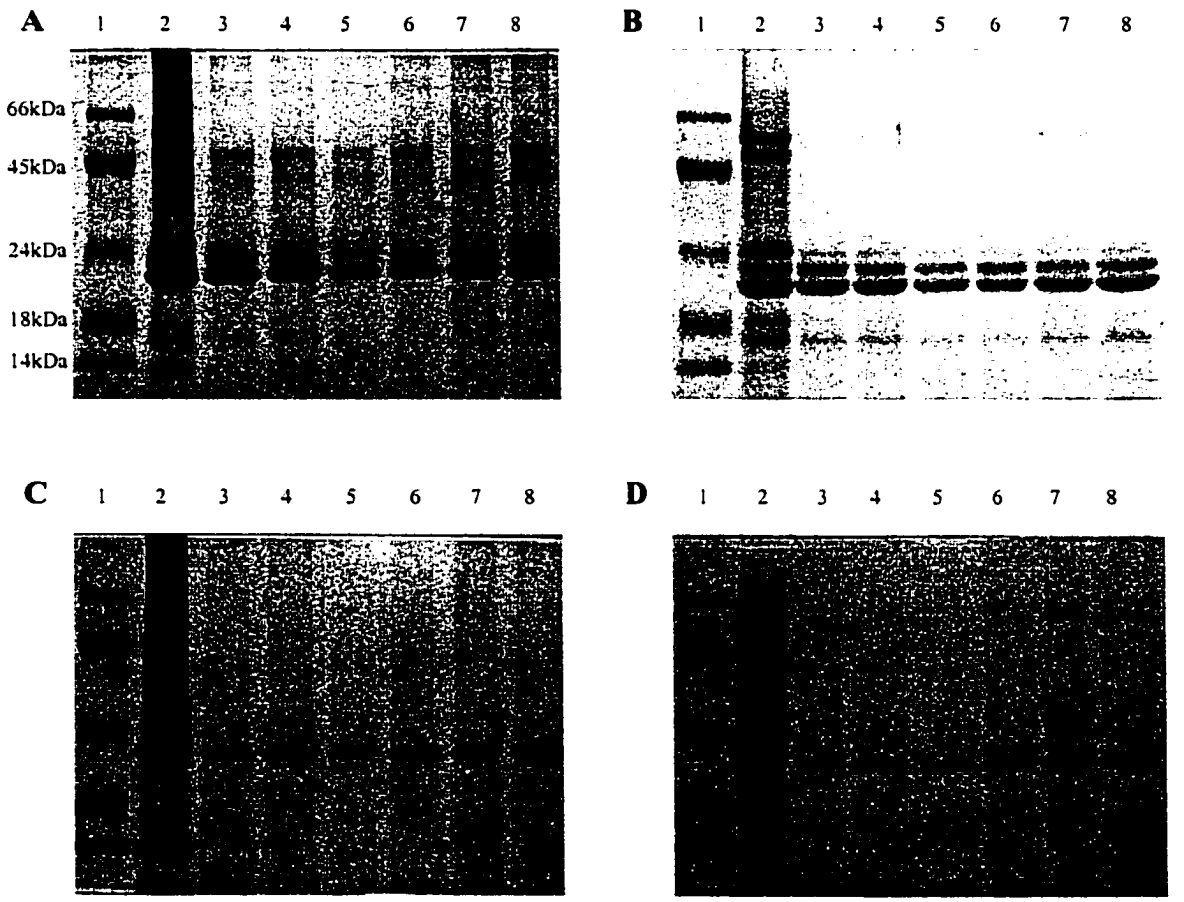

Figure 4.3 - SDS-PAGE patterns of proteins extracted from the unprocessed and extruded normal (A/B) and QPM (C/D) maize grits using 6M Urea in 10mM Tris buffer pH 8 at ambient temperature. Electrophoresed in non-reducing (A/C) and reducing (B/D) conditions in a mini-gel of 12.5% polyacrylamide running gel and 6% stacking gel. Run at 100V for 100 min. Running buffer 50mM Tris, 192mM glycine , 0.1%SDS. Lanes : 1) molecular weight marker, 2)unprocessed corn grits 3) grits extruded at SME =50, 4) SME=75, 5) SME =160, 6) SME =260, 7) SME =320, 8)SME =430.

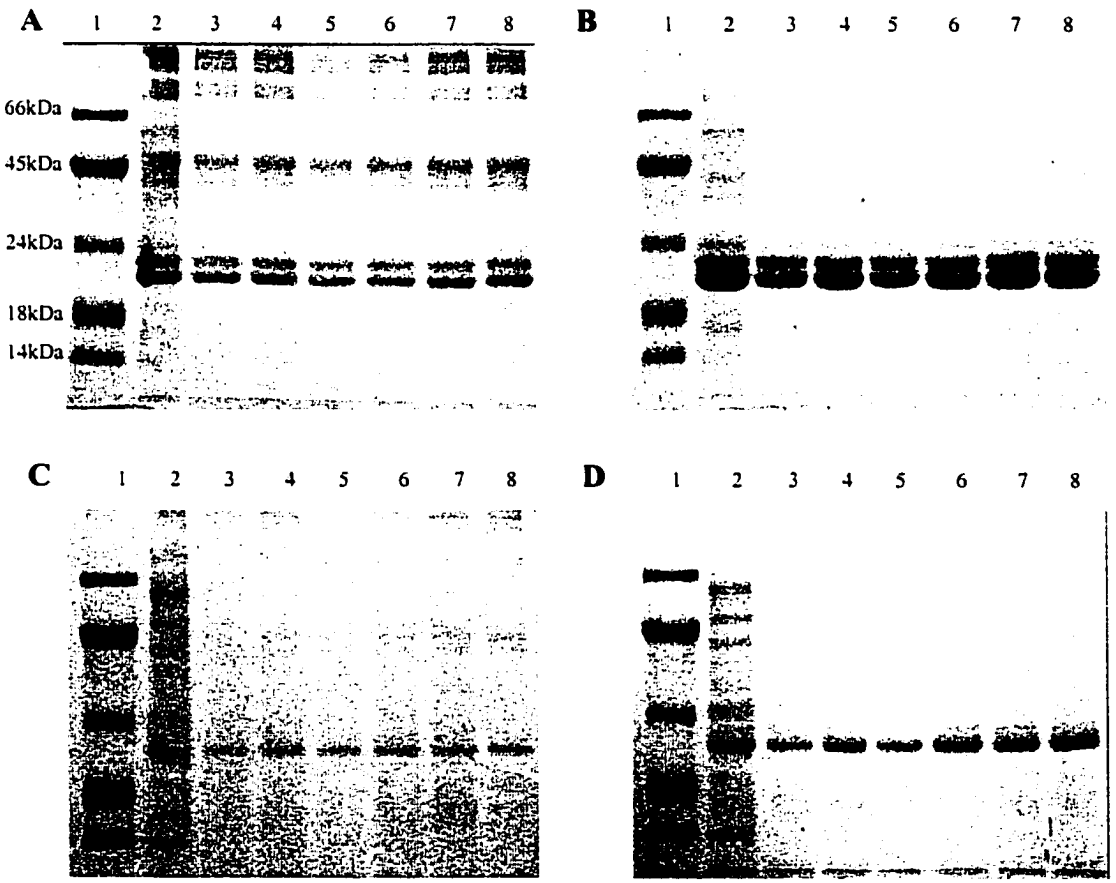

Figure 4.4 - SDS-PAGE patterns of proteins extracted from the unprocessed and extruded normal (A/B) and QPM (C/D) maize grits using 2% SDS in 10mM Tris buffer pH 8 at ambient temperature. Electrophoresed in non-reducing (A/C) and reducing (B/D) conditions in a mini-gel of 12.5% polyacrylamide running gel and 6% stacking gel. Run at 100V for 100 min. Running buffer 50mM Tris, 192mM glycine , 0.1%SDS. Lanes : 1) molecular weight marker, 2)unprocessed corn grits 3) grits extruded at SME =50, 4) SME=75, 5) SME =160, 6) SME =260, 7) SME =320, 8)SME =430.

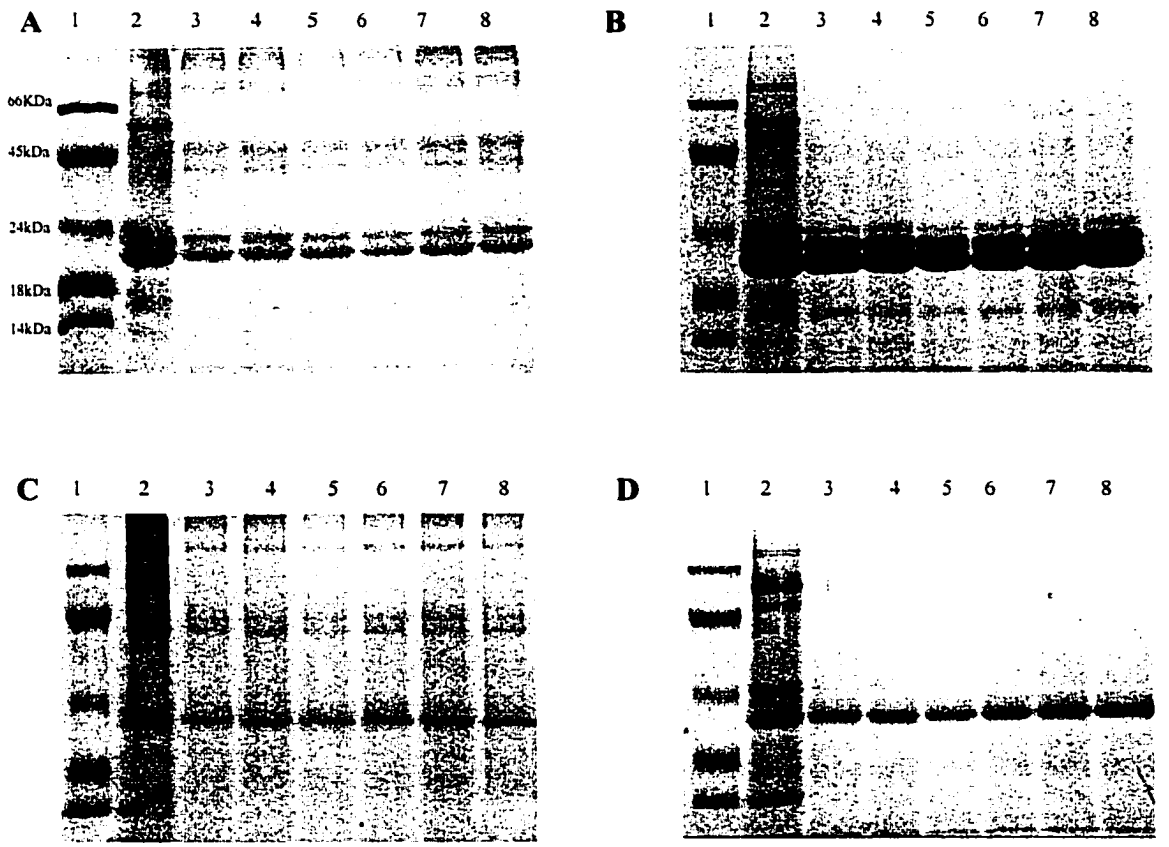

Figure 4.5 - SDS-PAGE patterns of proteins extracted from the unprocessed and extruded normal (A/B) and QPM (C/D) maize grits using 6M urea, 2% SDS in 10mM Tris buffer pH 8 at ambient temperature. Electrophoresed in non-reducing (A/C) and reducing (B/D) conditions in a mini-gel of 12.5% polyacrylamide running gel and 6% stacking gel. Run at 100V for 100 min. Running buffer 50mM Tris, 192mM glycine , 0.1%SDS. Lanes : 1) molecular weight marker, 2)unprocessed corn grits 3) grits extruded at SME =50, 4) SME=75, 5) SME =160, 6) SME =260, 7) SME =320, 8)SME =430.

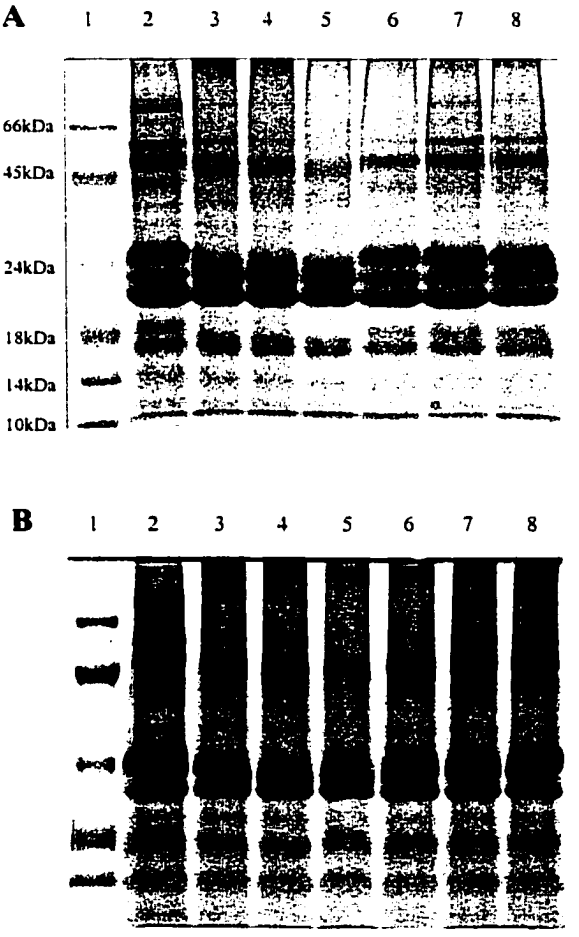

Figure 4.6 - SDS-PAGE patterns of proteins extracted from the unprocessed and extruded normal (A) and QPM (B) maize grits using 6M, urea, 2% SDS, 2% 2-ME in 10mM Tris buffer pH 8 at ambient temperature. Electrophoresed in reducing conditions in a mini-gel of 12.5% polyacrylamide running gel and 6% stacking gel. Applied 20μl of sample in each well. Run at 100V for 100 min. Running buffer 50mM Tris, 192mM glycine , 0.1%SDS.

Lanes : 1) molecular weight marker, 2)unprocessed corn grits 3) grits extruded at SME =50, 4) SME=75, 5) SME =160, 6) SME =260, 7) SME =320, 8)SME =430.

Warner-Bratzler test

Breaking strength

Breaking strength (g/mm^2) (BS) was determined in the extrudates in order to measure the maximum stress each sample can withstand before breaking. It was used as an indicator of hardness in extruded products (Hsieh *et al.* 1990; Chinnaswamy and Hanna 1988; Mathew and Hanna 1997), and also to determine sensory brittleness, following the findings of Sherman and Deghaidy (1978), who reported an inverse relationship between maximum force to fracture and brittleness in low moisture foods.

Analysis of variance of the BS data revealed that the three-way interaction and the two-way interactions for moisture/maize type and SME levels/maize type were not significant at $p<0.05$ (Appendix VIII). Consequently, data were reanalyzed and degrees of freedom for those interactions were combined into the degrees of freedom for the error term. New analysis provided significance for the SME levels and moisture interaction, allowing the comparison of breaking strength means averaged over maize type. These results are shown on Figure 4.7.

Brittleness of the maize extrudates was inversely related to moisture level regardless of maize type. Extrudates equilibrated to 16% moisture presented significantly lower brittleness ($p<0.05$) than those equilibrated at lower moisture levels, requiring more force per area to be ruptured. Perhaps, moisture acts as a plasticizer, leading protein and starch to glass transition, which resulted in reduced brittleness of extrudates with lower values for breaking strength

The combined effects of moisture with shear to brittleness could be only significantly perceived on samples equilibrated to 12 and 16% moisture, although the trend was also present in samples equilibrated at the lowest moisture level. Regardless of

moisture content, increases in energy input in the extruder produced extrudates with higher breaking strength, thus less brittle and hard. But, extrudates produced at the highest SME, apparently required less force to break than those produced at intermediate levels ($p \geq 0.05$). Increase in shear above 320 kJ/kg, usually applied in the production of corn-based extrudates, seemed to favor brittleness (crispness) ($p \geq 0.05$). Possibly low shear (SME < 75kJ/kg) did not provide energy input enough to melt all particles, therefore, some spaces among non-melted grits may have provided less resistance in breaking of the extrudates.

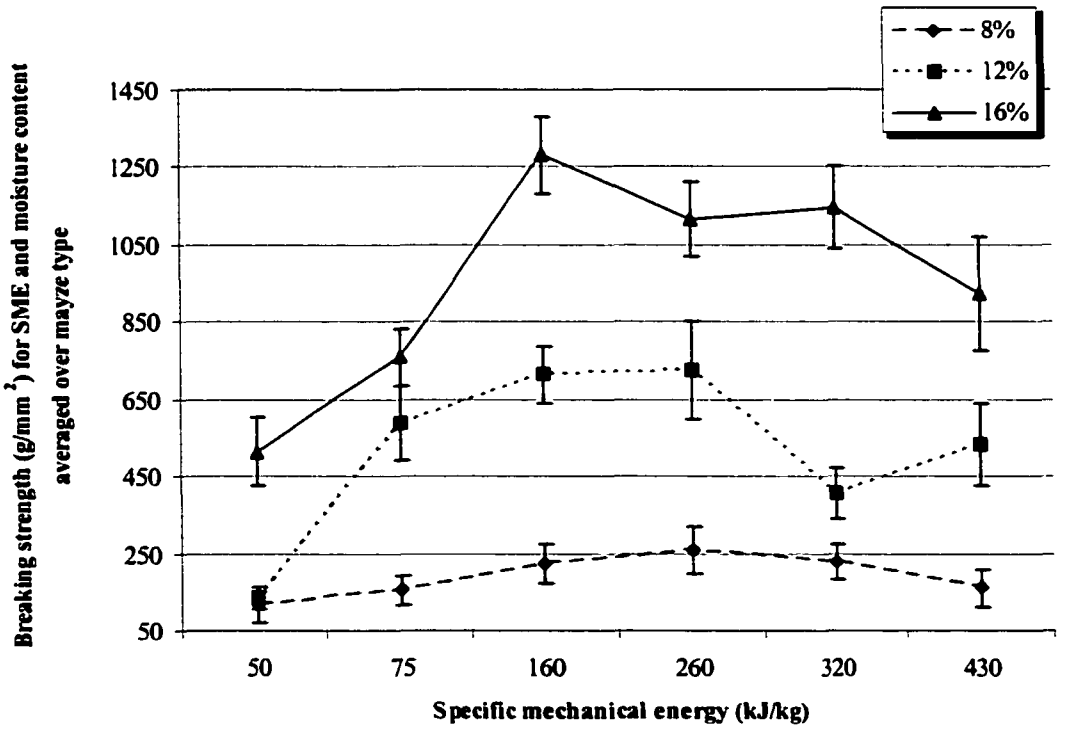

Figure 4.7. Breaking strength (g/mm2) of maize extrudates processed at various SME levels and conditioned at 8, 12, and 16% moisture contents.

In general, QPM extrudates were more brittle than normal maize extrudates, since the overall breaking strength mean for all QPM extrudates was significantly lower ($p<0.05$) than for normal maize extrudates (Fig. 4.8). In light of the fact that starch isolated from QPM and normal maize grits have shown similar functional properties (Chapter II), differences in extrudates brittleness are consistent with differences in protein composition of the unprocessed material. Moreover, highly disulfide bound zeins in QPM grits likely provided higher strength to its extrudates, thus favoring extrudate brittleness

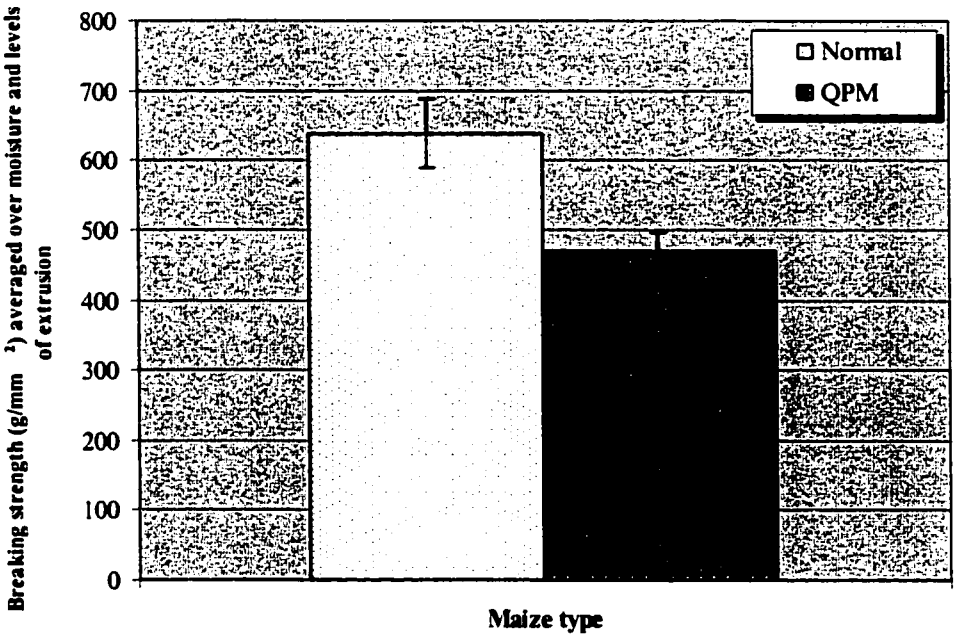

Figure 4.8. Breaking strength (g/mm2) of normal and QPM maize extrudates averaged over moisture and SME levels.

According to Sherman and Deghaidy (1978), a food product determined as crispy by sensory evaluation, requires a lower force (kg) to shear during objective evaluation

using an Instron UTM. Therefore, it may be inferred from our results that QPM extrudates are somewhat crisper than their counterparts.

CONCLUSIONS

Solubility of maize proteins in different solvents indicated that both noncovalent and covalent interactions are important in holding the structure of maize proteins. Also, it indicated that extrusion promotes modifications in the chemistry of maize proteins, mainly zeins. After extrusion at mid range of SME's, proteins become less soluble, perhaps due to protein aggregation. But harsh extrusion conditions (>320 kJ/kg) apparently favored the solubilization of zeins. In general, modifications in protein chemistry were driven by noncovalent and covalent interactions, although the disulfide bonding was apparently more important for the QPM samples. Chemical changes in proteins were identified as an important factor influencing brittleness of the final extrudates. Moisture negatively affected the brittleness of extrudates, regardless of maize type. Higher content of γ-zein in QPM likely provided higher strength to maize extrudates thus favoring crispness of extruded maize grits. Considering that moisture affected brittleness in the same way in the normal and QPM samples, the use of QPM grits to replace normal grits in the production of extruded corn-based products may promote extended bowl-life of extruded corn flakes as well as enhance crispness of fully expanded snacks.

GENERAL CONCLUSIONS

Based on the conditions used in this research, the following conclusions can be made:

1. Laboratory-isolated starch from QPM and normal maize grits are not different in regard to pasting properties, although cultivar is an important factor affecting hot paste viscosity and cool paste viscosity of maize starch.
2. Starch isolated from QPM and normal maize grits have somewhat dissimilar thermal gelatinization properties. Starch from QPM grits has slightly higher onset (T_o) and peak gelatinization temperature (T_p) than starch isolated from normal maize.
3. Maize grits produced with semi-flint QPM kernels contain higher content of γ-zein and reduced α-zein when compared to grits of a normal semi-flint maize cultivar.
4. Extrusion caused significant changes in the microstructural organization of starch granules, protein bodies, and the protein matrix identified in the maize grits. Starch granules melt and form a continuous matrix, whereas proteins aggregate into large masses diffused in the gelatinized starchy network.
5. The high γ-zein content in QPM seems to confer resistance to disruption of protein bodies during extrusion. Therefore, high γ-zein rich protein

bodies require higher energy input within the extruder for complete disruption than protein bodies found in normal maize.

6. Differences in dispersion of zeins in the system for the two maize types were not identified in the conditions of the study.

7. Extrusion promotes modifications in the chemistry of maize proteins, mainly zeins. After extrusion at mid range of SME's, proteins become less soluble, perhaps due to protein aggregation. But harsh extrusion conditions (>320 kJ/kg) apparently favor the solubilization of zeins.

8. Modifications in protein chemistry due to extrusion are driven by noncovalent and covalent interactions, although the disulfide bonding is apparently more important for the QPM samples.

9. Chemical changes in proteins resulting of extrusion were identified as an important factor influencing brittleness of the final extrudates.

10. Moisture negatively affected the brittleness of extrudates, regardless of maize type.

11. Higher content of γ-zein in QPM likely provided higher strength to maize extrudates thus favoring crispness of extruded maize grits.

12. The use of QPM grits to replace normal grits in the production of extruded corn-based products may promote extended bowl-life of extruded corn flakes and enhance crispness of fully expanded snacks.

RECOMMENDATIONS

Additional studies on fine structures and microstructures of starch isolated from normal and QPM corn genotypes would be useful to understand the differences in pasting properties among cultivars and, to elucidate the interaction of protein and starch in the endosperm of maize. The findings of these prospective studies might provide new insights for kernel hardness, an important characteristic not only for resistance to insects and microorganisms but also in the processing of maize through a dry-milling process.

Additional techniques, including laser confocal scanning microscopy combined with staining and immunocytochemistry, and reaction with specific fluorescent dyes would helpful in assessing changes in morphology of starch granules and protein bodies, as well as zein dispersion in extruded samples. Light microscopy could also be used to assess the distribution of protein aggregates in the extruded samples.

Production of corn-meal and isolated zein following the standard procedures applied in the food industry would allow for testing of the γ-zein rich prolamin fraction in QPM for further applications such as in biodegradable packaging materials and plastic films.

To confirm the relevance of zein composition in the textural properties of extruded corn-based products, including crispness and bowl-life, it would be important to design experiments where final products, such as fully expanded corn-curls and extruded cornflakes would be produced using the same ingredients applied in the large scale processing of these products. The combination of subjective and objective evaluation should be conducted in these products.

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APPENDICES

APPENDIX I

PROTEIN FRACTION PERCENT DISTRIBUTION IN DIFFERENT MAIZE TYPES

	Normal	Opaque-2	QPM
		%	
Albumin + globulin	6.2	20.6	15.5
Prolamin (zein)	39.2	8.1	10.4
Prolamin-like	19.7	10.7	16.2
Glutelin	22.7	42.5	36.6
Glutelin-like	13.6	18.5	21.4

Source: Serna-Saldivar and Rooney (1994)

APPENDIX II

AMINO ACID COMPOSITION OF ZEIN POLYPEPTIDES

	α- zein M_r 19,000	α- zeins M_r 22,000	γ-zein M_r 27,000	γ-zein M_r 16,000	β-zein M_r 15,000	δ-zein M_r 18,000	δ-zein M_r 10,000,
	mol%						
Asn	4.5	5.3	0.0	0.6	1.9	1.1	2.4
Asp	0.5	0.0	0.0	0.0	0.6	0.5	0.8
Thr	3.2	2.8	4.4	3.7	2.5	5.9	3.1
Ser	7.3	6.9	3.9	5.5	5.1	9.1	6.3
Gln	19.5	20.7	14.7	18.9	16.5	8.6	11.8
Glu	0.5	0.8	1.0	1.8	1.9	0.5	0.0
Pro	10.0	8.9	25.0	15.2	8.9	17.2	15.7
Gly	0.9	0.8	6.4	9.1	8.9	2.7	3.1
Ala	13.6	13.8	4.9	7.9	13.9	5.4	5.5
Cys	0.9	0.4	7.4	7.3	4.4	1.6	3.9
Val	2.7	6.9	7.4	4.9	2.5	3.2	3.9
Met	0.5	2.0	0.5	1.8	11.4	26.9	22.8
Ile	4.5	4.5	2.0	0.6	0.6	4.8	4.7
Leu	19.5	17.1	9.3	8.5	10.1	5.4	9.4
Tyr	3.6	2.8	2.0	4.9	8.9	1.6	0.8
Phe	5.5	3.3	1.0	4.3	0.0	2.7	3.9
His	0.5	1.2	7.8	2.4	0.0	1.1	1.6
Arg	1.4	1.6	2.5	1.8	3.2	0.0	0.0
Lys	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.5**	**0.0**
Trp	**0.0**	**0.0**	**0.0**	**0.6**	**0.0**	**1.1**	**0.0**

Source: Coleman and Larkins, 2000

APPENDIX III

CHEMICAL COMPOSITION OF DIFFERENT TYPES OF MAIZE

	Dent	Flint[3]	Popcorn[3]	Flour[3]	Opaque-2	QPM
Moisture (%)	16.0[2]	-		-	-	12.0[5]
Starch (%db)	71.7[2]	11.1	62.3	-	64.4[3]	71.0[4]
Protein(%db)	9.5[2]	4.7	11.9	-	13.6[3]	10.5[1]
Fat (%db)	4.3[2]		5.3	-	5.5[3]	5.1[5]
Fiber (%db)	9.5[2]	-	2.6	-		2.2
Sugar (%db)	2.6[2]	-	9.3	-	2.8[3]	-
Ash (%db)	1.4[2]	-	1.9	-	-	1.5[5]
Lysine (g/100g protein)	2.7[2]	2.0	2.0	2.7		3.9[1]
Tryptophan (g/100g protein)	0.59[4]	-	-			0.75[1]

[1] Guimaraes *et al.*(1994)
[2] Watson (1987)
[3] Johson (1991)
[4] Serna-Saldivar and Rooney (1994)
[5] Paes and Bicudo (1994)
[6] Mertz (1992)

APPENDIX IV

ADDITIONAL DATA FROM THE EXTRUSION PROCEDURE

Maize cultivar	Replicate	Moisture %	Screw type	Screw speed (RPM)	Torque (NM)	Feed rate	SME (kJ/kg)
BR 473	1	22	1:1	40	10.7	50.6	53.1
BR 473	2	22	1:1	40	10.8	48.2	56.3
BRS 3143	1	22	1:1	40	12.2	56.4	54.4
BRS 3143	2	22	1:1	40	11.5	54.2	53.3
BR 473	1	22	1:1	40	15.6	50.3	77.9
BR 473	2	22	1:1	40	13.6	45.3	75.5
BRS 3143	1	22	1:1	40	11.9	41.8	71.6
BRS 3143	2	22	1.1	40	15.7	51.7	76.3
BR 473	1	22	3:1	20	28.1	22.6	156.3
BR 473	2	22	3:1	20	29.9	22.9	163.8
BRS 3143	1	22	3:1	20	29	22.4	162.7
BRS 3143	2	22	3:1	20	28.8	22.5	160.9
BR 473	1	22	dispersive	60	32.1	46.5	260.3
BR 473	2	22	dispersive	60	32.1	53	266.4
BRS 3143	1	22	dispersive	60	33.1	46.7	267.2
BRS 3143	2	22	dispersive	60	32.9	48.1	257.6
BR 473	1	22	dispersive	60	45.4	52.3	327.3
BR 473	2	22	dispersive	60	44.7	51	330.2
BRS 3143	1	22	dispersive	60	43.1	52.3	310.7
BRS 3143	2	22	dispersive	60	43.6	52.3	314.0
BR 473	1	22	3:1	60	32.5	28.8	425.9
BR 473	2	22	3:1	60	34.2	29.7	434.1
BRS 3143	1	22	3:1	60	34.7	29.8	438.9
BRS 3143	2	22	3:1	60	34.8	29.9	438.8

APPENDIX V

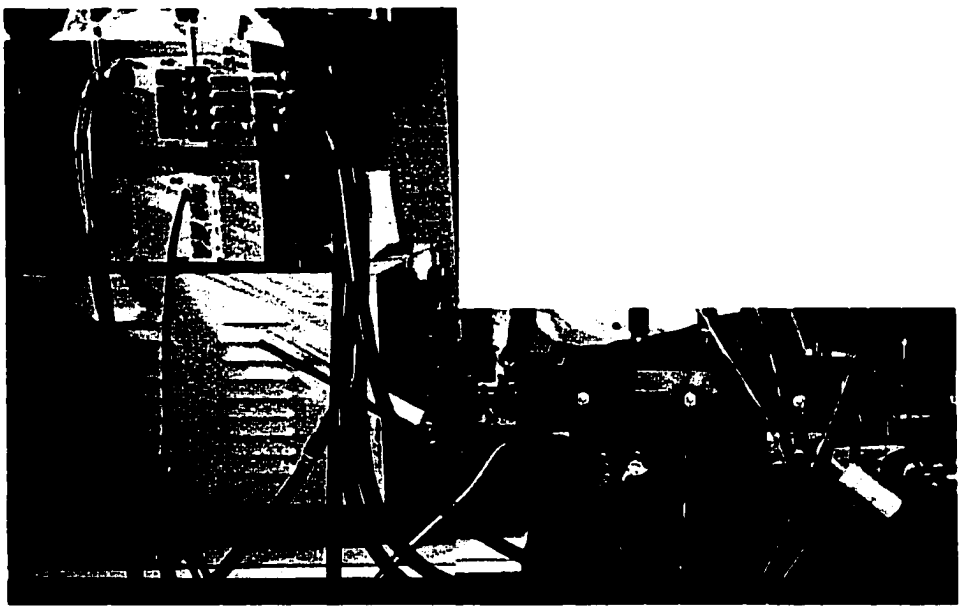
DESIGN OF THE DISPERSIVE AND 3:1 SCREW TYPES USED IN THE EXTRUSION OF MAIZE GRITS

Dispersive

3:1

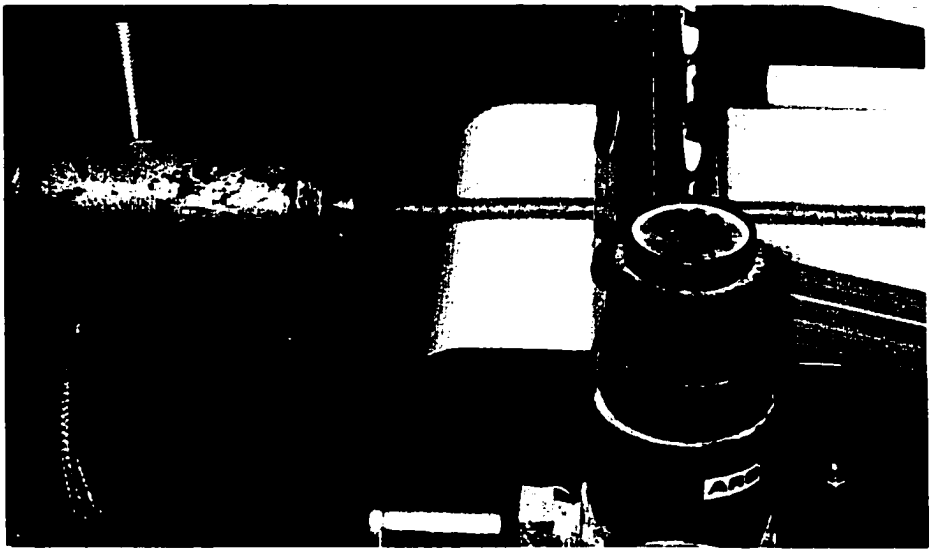
APPENDIX VI

EXTRUDER USED IN THE PROCESSING OF MAIZE GRITS



APPENDIX VII

EXTRUDED MATERIAL EXITING THE DIE OPPENING DURING EXTRUSION OF MAIZE GRITS



APPENDIX VIII

RESULTS OF ANALYSIS OF VARIANCE (ANOVA) PERFORMED FOR BREAKING STRENGHT AFTER POOLING NON SIGNIFICANT FACTORS INTO ERROR TERM

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
Maize type	1275798.348	1	1275798.348	20.365	0.000
SME	4411424.464	5	882284.893	14.084	0.000
Moisture	1.75926E+07	2	8796316.001	140.414	0.000
SME* moisture	2124905.828	10	212490.583	3.392	0.000
Error	1.00233E+07	160	62645.399		