1995

Effects of ear developmental temperature on fine structure of maize starch

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Effects of ear developmental temperature on

fine structure of maize starch

by

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A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Food Science and Human Nutrition
Major: Food Science and Technology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

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Signature was redacted for privacy.

Signature was redacted for privacy.

Signature was redacted for privacy.

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Iowa State University
Ames, Iowa

1995

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ACKNOWLEDGMENTS

I would like to express my sincere gratitude to Dr. Jay-lin Jane for her advice, guidance and encouragement, during the pursuit of my degree at Iowa State University. Special thanks are to Dr. Dennis C. Johnson for his advice and kindly allowing me to use his equipment during my studies on the HPAEC-PAD.

I also want to extend my appreciation to the members of my committee, Dr. Lawrence A. Johnson, Dr. Zivko Nikolov, Dr. Linda M. Pollak, Dr. John F. Robyt and Dr. Pamela J. White for their interest and valuable help.

GENERAL INTRODUCTION

Starch is the storage polysaccharide of most plants. It consists of a mixture of two similar polymers of glucose: the essentially linear amylose and the branched amylopectin. The starch of most plants contains about 20-25% amylose and 75-80% amylopectin, but this ratio is not fixed.

The starch biosynthesis pathway is complicated and not completely understood. Although gross starch structure is similar in various species, the fine structure and granule characteristics still need further study (Beck and Ziegler, 1989; Boyer, 1985; Preiss, 1982; Preiss and Levi, 1980). Variations are associated with plant species, cultivars of a species, genetic mutations, growth stages, and the environment in which the plant is grown.

Temperature is one environmental variable that cannot be manipulated in the field, and crops are often selected for a region on the basis of their response to the temperature ranges and growing season of that region. Moreover, temperature fluctuations have a great influence on both the quantity and quality of starch biosynthesis. Changes in environmental growth temperature and other climatic factors are responsible for variations in starch properties. This variation directly affects the grain quality and value, and is carried over as a quality control problem in some products.

Effects of environmental temperature on cereal crops have been reported. For example, high temperature (25/15°C and 35/25°C, day/night temperature) reduces whole

maize plant dry matter accumulation during grain filling (Badu-Apraku et al.,1983). High temperature also significantly decreases amylose content in the rice (Asaoka et al., 1984) and maize starch of high-amylose strains (Fergason and Zuber, 1962) and changes fine structure of rice amylopectin (Asaoka et al.,1985). Development rate of maize does not follow a linear correlation with the temperature scale. Developmental response to temperature is not the same in all subperiods nor is it the same for all cultivars. Experimental results have shown that temperature fluctuations and the timing of these fluctuations are also important for both grain development and yield (Brown, 1977). However, despite the importance of growing temperature effects on grain growth, how temperature affects the biosynthesis of maize starch structures has not been extensively studied.

Although the mechanism of temperature affecting the starch biosynthesis pathway is still not clear, the effects, perhaps, can provide an insight to understanding the regulation of starch biosynthesis. The objectives of the present study are to investigate how growth temperature affects the structure and functional properties of maize starch and how the temperature affects on branch chain formation during starch biosynthesis.

Dissertation Organization

This dissertation consists of three papers. The first paper entitled "Separation and Quantification of Malto-oligosaccharides by High-Performance Anion-Exchange

Chromatography with Pulsed Amperometric Detection" was an analytical method development for studying starch structure, and will be submitted for publication to the journal Carbohydrate Research. The second paper, "Effects of Ear Developmental Temperature on Maize Starch Fine Structure" was submitted for publication to the journal Carbohydrate Research. The third paper, "The Temperature Effect on Retrogradation of Amylose Solution," will be submitted to the same journal. The three papers follow the format of the journal Carbohydrate Research and are preceded by a General Introduction and followed by a General Conclusion. Literature cited in the General Introduction are listed in alphabetical order according to author's name at the end of this chapter.

Literature Review

Starch is a mixture of glucans which is found mainly in the plant kingdom. Starch occurs as the principal energy reserve polysaccharide and may be used during growth of the plant. Since starch forms the major source of carbohydrate in the human diet, starch is of great economic importance, especially in food manufacture. Starch, modified starch, and starch derivatives have many uses in industry and the applications, and industrial demand of starch are growing. The history of starch in the development of the food and chemical industry has been reviewed (Whistler, 1965; Whistler, 1984).

Occurrence and morphology of starch

The main sources of starch are the higher plants, where the polysaccharide is laid down in the insoluble form of granules, although starch-like material also has been observed in some bacteria, protozoa, and algae (Greenwood, 1970; Shannon and Garwood, 1984). Starch is found in all parts of the plant, leaves, stem, shoots, and storage organs such as tubers, rhizomes, and seeds (Greenwood, 1970; Shannon and Garwood, 1984). The proportion varies from a few percent to 80% (*e.g.* grains of cereal) (Greenwood, 1970). The morphology of the starch granule is characteristic of the botanical source. According to the way they are deposited in the organelle, starch granules may be classified as (1) single granules, *i.e.* wheat and corn, which grow as a single granule inside an amyloplast or chloroplast; (2) compound granules, *i.e.* rice and oat, in which many starch granules grow within a single amyloplast; (3) semi-compound granules, *i.e.* amaranth, which initially grow as two or more distinct granules and then fuse together; or (4) pseudo-compound granules, *i.e.* wrinkled pea, which grow as individual granules but which, upon drying, develop large cracks and split into apparent compound granules (Lineback, 1984; Shannon and Garwood, 1984). Starch size ranges from the sub-micrometer or several μm (*e.g.* amaranth, 0.5-2 μm; taro, 2-4 μm; and rice, 3-8 μm) to over 100 μm (canna) in diameter (Fitt and Snyder, 1984; French, 1984; Jane et al., 1992; Jane et al., 1994). The shapes of granules are diverse, *i.e.* disc, elliptical, lenticular, round, oval, polygonal etc. Most granules appear to be built up in layers which partially or completely encircle the hilum, the original growth point. The hilum is usually less organized than the rest of the granule (French, 1984; Lineback,

1984). The hilum position varies from centric (wheat) to extremely eccentric (banana) (Seidemann, 1966; Wivinis and Maywald, 1967; Czaja, 1969; Fitt and Snyder, 1984; French, 1984; Jane et al., 1994). Starch granules are birefringent in polarized light and exhibit the Maltese Cross pattern ranging from very brilliant (potato) to relatively weak (wheat) (Wivinis and Maywald, 1967; Fitt and Snyder, 1984).

Organization of starch granule

The heterogeneity of the starch granule, a mixture of linear and branch components, was experimentally proven by Maquenne and Roux (Maquenne, 1904; Maquenne and Roux, 1905). The two major components are amylose (the linear polymer) and amylopectin (the branched polymer). The amylose and amylopectin ratio varies with botanical source of the starch and greatly affects the characteristics of starch granules. The amylose content ranges from a trace amount (waxy rice and maize) to 70 % or higher (wrinkled-seeded pea and amylomaize) (Young, 1984; Kennedy et al., 1987). Most normal starch granules contain 20-30% amylose. Besides the two major components, Lansky et al. (1949) postulated that maize starch contained about 5-7% of a material, called intermediate fraction, having different properties from amylose and amylopectin. Although this intermediate fraction can be fractionated (Whistler, 1964; Banks and Greenwood, 1975; Takeda and Preiss, 1993; Wang et al., 1993) there have been no critical definition of this fraction.

How the starch components are organized in the granules is not yet fully understood. In the late 19th century, a magnificent trichitic model (Figure 1) was

proposed by Meyer (1895). Lineback (1984) commented on the model, "The concentric ring concept is most remarkable because the concept of high polymers had not, then, been developed. Further, it was not then known that starch contained two major polysaccharide components." In 1969, Nikuni proposed a model of the starch granule in which all molecules were covalently bound together (Figure 2) (Nikuni, 1969; Nikuni, 1978). It is unlikely that amylose is covalently bound to amylopectin because of its ease of leaching. This model was modified by Lineback in 1984 (Figure 3). The modified model incorporated the concept of double helices in the outer chains of amylopectin, a helical amylose-lipid complex, and random coil conformations of amylose. According to the observation and investigation of the growth ring of the starch granule, an arrangement of the amylopectin molecules within a growth ring was proposed by Kainuma (1980) (Figure 4). The growth ring resulted by alternating high and low refractive index layers, which also differ in density, crystallinity, and resistance or susceptibility to chemical and enzymic attack (French, 1984; Lineback, 1984). The growth ring apparent layers represented the internal features of molecular arrangement in starch granules. The molecules in the granule are arranged in an ordered radial manner, thus resulting in its birefringent properties. The high density, crystallinity, and resistant layers were proposed as the highly ordered-alignment portion of amylopectin (Biliarderis, 1981).

Recent investigations have provided more details to the structure of the starch granule. The location of amylose has been confirmed by using cross-linking reactions, and was shown to be interspersed among amylopectin and not present in bundles (Jane

et al., 1992; Jane et al., 1993; Kasemsuwan and Jane, 1994). Furthermore, the amylose content in the starch granule is more concentrated at the periphery of the granule (Jane and Shen, 1993). A starch crystallite model was proposed for the disposition of amylopectin, amylose and fatty acids in the maize granule by Zobel (1992) which showed the amylose molecules incorporated in amylopectin crystallites and amylose-fatty acid complexes (Blanshard, 1987).

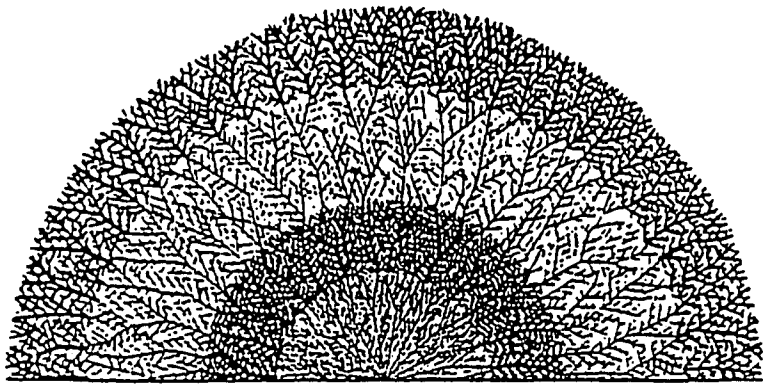

Figure 1. Trichitic model of starch granule proposed by Meyer (1895).

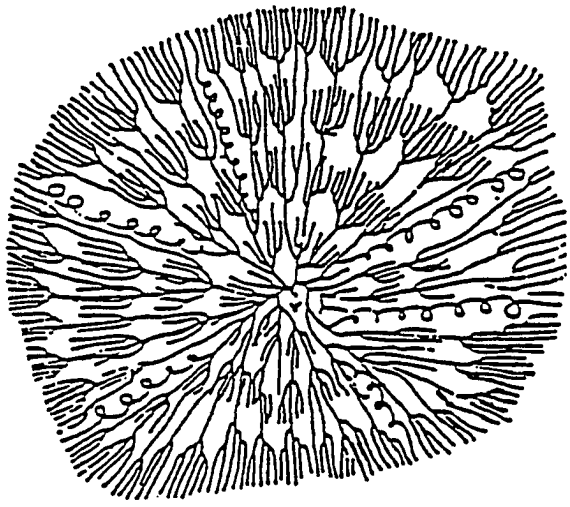

Figure 2. Schematic representation of the organization of a starch granule proposed by Nikuni (1969).

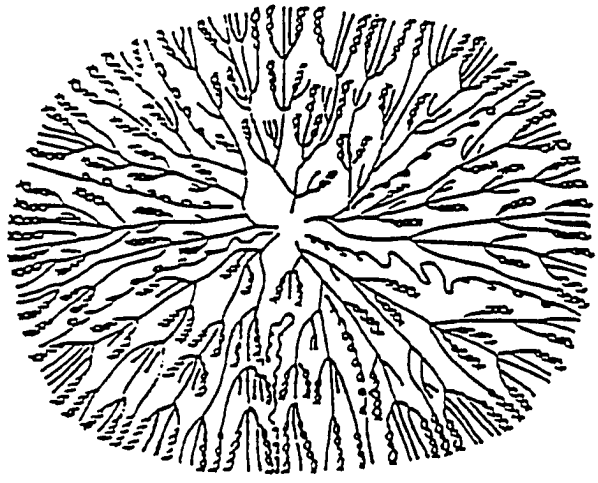

Figure 3. Schematic representation of the organization of a starch granule proposed by Lineback (1984).

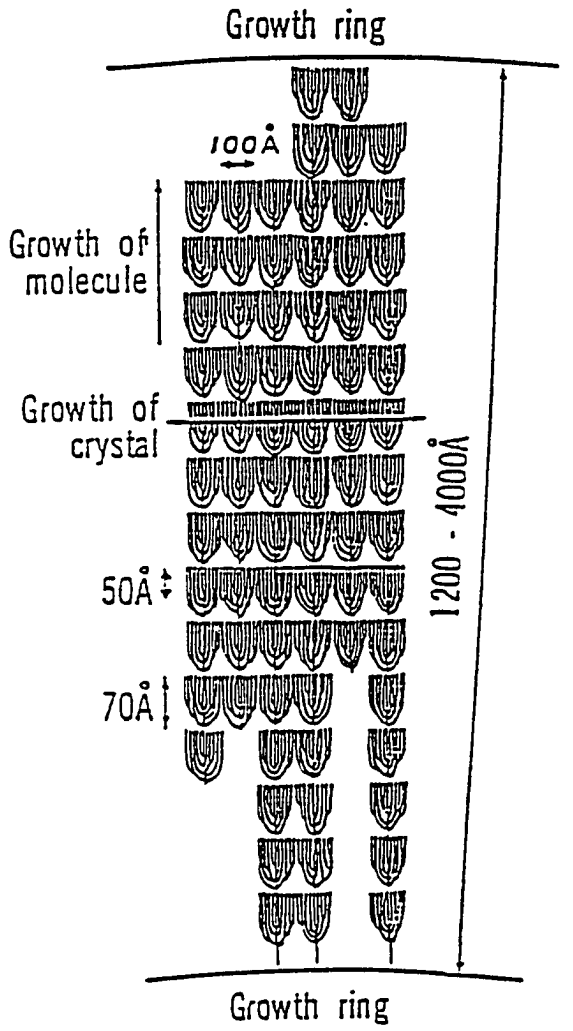

Figure 4. Schematic representation of the arrangement of amylopectin molecules in a waxy maize starch granule proposed by Kainuma (1980).

Nature of amylose

Amylose is an essentially linear α-D-glucopyranose polymer linked by α-(1→4) linkages bonds with a 500-5,000 degree of polymerization (Greenwood, 1970; Galliard and Bowler, 1987). The linear nature of amylose was generally accepted until Peat et al. (1949, 1952) showed that crystalline sweet potato β-amylase only hydrolyzed 70% of the amylose to maltose. Using data for the hydrodynamic radius of amylose and multiple enzymic hydrolysis, Banks and Greenwood (1966 and 1967) showed that amylose contains some branch points. Hizukuri et al. (1981 and 1983) confirmed the multi-branched nature of amylose from several plant sources. Recently, direct structure analytical evidence also was provided by Cura et al. (1995). Amylose, like other linear polymers, is prompt to retrograde. In aqueous solution, amylose molecules rapidly associate to build up molecular aggregates that soon exceed colloidal dimensions and precipitate (Whistler, 1965; Miles, et al., 1984; Colonna et al., 1992). The aggregation process is rapid, and the molecular size is too large to produce perfect crystallites (Flory, 1953; Greenwood, 1964). As a result, the precipitate is a mixture of crystalline and amorphous regions as indicated by X-ray diffraction patterns and acidic or enzymatic hydrolysis (Kainuma and French, 1971; Colonna et al., 1992; Cairns et al, 1995). Retrograded amylose precipitate shows an A- or B-type X-ray pattern that depends on the retrogradation conditions (Hizukuri, 1964; Wu and Sarko, 1978; Kitamura et al., 1984; Buleon et al., 1984; Ring et al., 1987; Eerlingen et al., 1993; Le Bail et al., 1993; Cairns et al., 1995). The crystalline region can be 30 to 65% of the total amylose gel, and the amount differs with the retrogradation conditions (Jane and

Robyt, 1984; Cairns et al., 1995). This crystalline region is resistant to acidic and enzymatic hydrolysis (Kainuma and French, 1971; Kainuma et al., 1981; Jane and Robyt, 1984, Tsuge et al., 1991; Cairns, 1995). The conformation of the resistant crystalline region was proposed to be double helices interspersed with amorphous regions (Wu and Sarko, 1978; Kodama et al., 1978; Jane and Robyt, 1984) similar to the starch chains in starch granules (Kanuma, and French, 1971; Kainuma and French, 1972; Yamaguchi et al., 1979; Oostergetel and van Bruggen, 1993). The chain length of the crystalline region from different studies varies (Jane and Robyt, 1984; Eerlingen et al., 1993; Cairns et al., 1995).

The stability of an amylose aqueous solution is dependent on molecular size, temperature, concentration, pH, and the presence of other chemical agents in the solution (Whistler, 1953; Young, 1984; Ellis and Ring, 1985; Suzuki et al., 1985). It has been shown that amylose from different starch sources retrograde at different rates, depending upon their average molecular weight (Whistler and Johnson, 1948; Whistler, 1953; Suzuki et al., 1985; Orford et al., 1987; Eerlingen et al., 1993). Amylose with DP 80 to 100 has the highest retrogradation tendency (Pfannemuller et al., 1971; Pfannemuller, 1986; Gidley et al., 1986; Gidley et al., 1989). Temperature is negatively correlated with retrogradation rate (Whistler, 1953). Suzuki et al. (1987) reported that an increase of retrograding temperature (0 to 30°C) of soluble starch solution resulted in a increase of the thermal transition temperature of retrograded starch in water. High pH deprotonates the hydroxyl group of amylose, and charge repelling inhibits the retrogradation process. Sugars increase retrogradation rate, whereas salts affect the rate

of retrogradation differently, depending on the salt species and concentration (Whistler, 1953, Loewus and Briggs, 1957; Germani et al., 1983; I'Anson et al., 1990; Eerlingen et al., 1994; Biliaderis and Prokopowich, 1994; Le Botlan and Desbois, 1995; Prokopowich and Biliaderis, 1995). Complex formation of amylose with iodine and hydrocarbon compounds is also a well known characteristic of amylose (Schoch, 1942; Whistler, 1965; Kuge and Takeo, 1968; Pfannemuller et al., 1971; Banks and Greenwood, 1975; Handa and Yajima, 1980; Handa et al., 1980; Handa et al., 1981). Surfactants, lipids and long chain alcohols can form complexes with amylose and prevent or inhibit retrogradation. (Schoch, 1942; Lansky et al., 1949; Kuge and Takeo, 1968; Whittam et al., 1986; Hibi and Kuge, 1987; Gudmundsson and Eliasson, 1990; Eelingen et al., 1994).

Nature of amylopectin

Amylopectin is a branched α-D-glucopyranose polymer linked by α-(1→4) linkages and about 5% α-(1→6) branch linkages with a 100,000 or larger degree of polymerization. The average branch-chain length is 20 to 25 (Whistler and Daniel, 1984; Manners, 1985). For high-amylose maize starch, the branch-chain length of amylopectin is above 30 (Hizukuri et al, 1983; Hizukuri, 1985, Jane and Chen, 1992). Long branch-chain length of amylopectin results in higher gelatinization temperature of starch granules (Jane et al, 1992), higher gel strength of starch paste, and higher turbidity of starch solution (Jane and Chen, 1992). A polymodal distribution of the amylopectin branch chain was shown by Hizukuri (1986). The branch chain length of

amylopectin was correlated with the crystalline properties of starch granule. A-type starch has shorter branch-chain length than B-type starch (Hizukuri et al., 1983; Hizukuri, 1985, Chen and Jane, 1994). The chains of amylopectin are categorized into three types: (1) A-chains are those that are linked to the molecule only by their reducing ends; (2) B-chains are those that are linked to the molecule by their reducing ends but, in addition, are branched at C-6 position in one or more of their D-glucopyraonsyl residues; and (3) C-chain is the one that bears the reducing end group (Peat, 1956). The ratio of A- to B-chain is an important characteristic of amylopectin structure (Enevoldsen and Juliano, 1988). The A:B chain ratio is calculated from the amount of maltose and maltotriose liberated from β-amylolysis limit dextrin by pullulanase (Peat et al., 1956; Marshall and Whelan, 1974; Umeki and Yamamoto, 1977; Asaoka et al., 1985). Manners (1985) summarized the results of previous studies and concluded that the A:B chain ratio of amylopectin ranges from 1.1 to 1.5. Several models have been proposed for amylopectin and extensively reviewed (Wolfrom and Khadem, 1965; Banks and Greenwood, 1975; Whistler and Daniel, 1984). The current, most adapted model is the cluster structure model (Figure 5) which was first proposed by Nikuni (1969, 1978) and sequentially modified by French (1972) and Robin et al. (1974) (Figure 5). Hizukuri (1986) based the further classification of the B chains, B1 to B4, on the observation of polymodal distribution of amylopectin branch-chains; the B chains were also classified into Ba and Bb chains on the basis of whether A chains were bound (Ba) or not bound (Bb) (Hizukuri and Maehara, 1990; Hizukuri and Maehara, 1991).

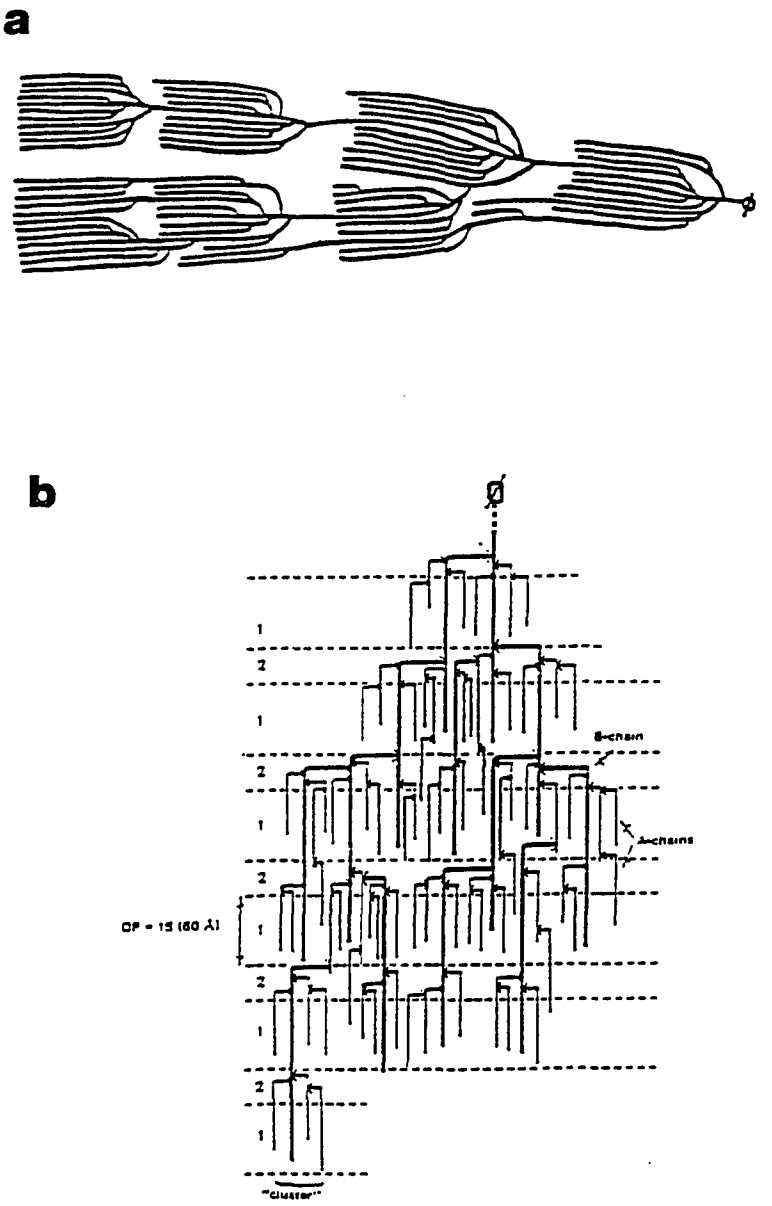

Figure 5. Cluster model for amylopectin structure proposed by (a) French (1972) and (b) Robin et al. (1974).

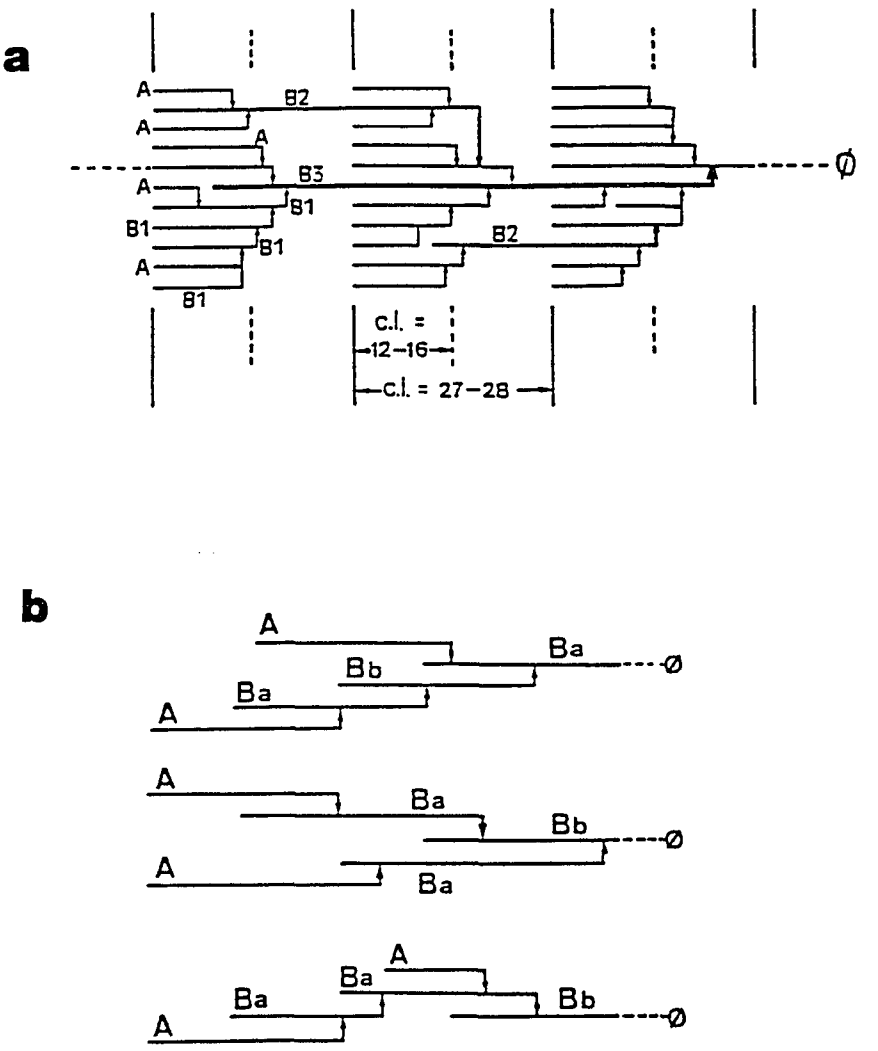

Figure 6. A cluster model for amylopectin with B-chain classification designated by (a) Hizukuri (1986) and (b) Hizukuri and Maehara (1990).

Starch biosynthesis

Starch biosynthesis involves three distinct enzymic processes: initiation, chain elongation and branching (Beck and Ziegler, 1989; Boyer, 1985; Preiss, 1982; Preiss and Levi, 1980). It has been proposed that a priming molecule, e.g. malto-oligosaccharides or glycoprotein, is required for initiating the biosynthesis of the starch chain, although this concept is currently the subject of some debate. Elongation of amylose and amylopectin outer branches occurs by the action of phosphorylase, starch-granule bound starch synthase, and soluble starch synthase. Phosphorylase uses glucose-1-phosphate as the substrate to transfer a glucopyranosyl unit onto amylose chain. Bound starch synthase, bound to the granule presumably to the amylose component, uses either adenosine diphosphate glucose (ADPG) or uridine diphosphate glucose (UDPG) as the glucosyl unit donor, but prefers ADPG. Soluble starch synthase, however, exclusively uses ADPG as the gulcosyl donors. The waxy cereals, which do not produce amylose, are also lacking bound starch synthase. The starch synthases elongate starch chains by joining D-glucopyranosyl units to the non-reducing ends of amylose and amylopectin on the granular surface. When the outer chains of amylopectin become sufficiently long, branching enzyme transfer a α-1,4 linkage to α-1,6 linkage. Unlike synthase, branching enzyme has little affinity for starch and can easily wash off from the granular surface (French, 1984).

Influence of environmental temperature on crop yield

The effect of temperature on kernel development and mature kernel weight of some cereals, such as sorghum, rice, and wheat, has been studied by Chowdhury and Wardlaw (1978). Wheat and sorghum showed clear and well-defined optimum temperatures of 15/10°C (day/night temperature) and 27/22°C, measured by individual kernel dry weights, whereas rice showed a relatively small change in weight over the temperature range from 21/16°C to 30/25°C. As temperature increases, the duration of grain filling for all of the cereals investigated is reduced. Grain weight of wheat decreases when the temperature exceeds 20°C, because the increased grain filling rate is not sufficient to compensate for the reduced filling duration (Sofield et al., 1977). A comparison of the effects of high temperature on grain development in wheat and rice has been reported by Tashiro and Wardlaw (1989). Weight of matured grain of the wheat cultivar Banks was reduced by about 5% for each 1°C rise in daily mean post-anthesis temperature in the range from 17.7 to 32.7°C, using grain weight at 17.7°C as the base. In contrast, the rice cultivar Calrose had stable grain weight up to 26.7°C. Above that temperature a 4.4% drop in weight per 1°C increase in mean post-anthesis temperature up to 35.7°C was observed. Grain weight at 26.7°C was used as the base. In both wheat and rice there was a reduction in the duration of grain growth as temperature increased up to a mean of 26.7°C. In this range rice, but not wheat, showed a compensating increase in the rate of dry-matter accumulation. Above 26.7°C the rate of dry-matter accumulation fell in both species, although this was more stable in rice than in wheat. In wheat the duration of grain growth continued to decrease at

temperatures above 26.7°C up to 35.7°C. However there was only little change in rice up to 35.7°C (Tashiro and Wardlaw, 1989).

This phenomena was also observed for potato tuber growth (Awan, 1964). Soil temperature decreased by mulching resulted in a significant increase in potato yields.

Most of the decreased weight at elevated temperature in wheat is attributed to a reduction in the amount of starch deposited during grain filling. Moreover, the number of small size starch granule (<10 um) was substantially reduced as temperature increased, but this reduction did not completely account for the smaller weight of starch per grain resulting from elevated temperature (Bhullar and Jenner, 1985).

Influence of environmental temperature on starch deposit

When the temperature is above the optimum for growth of cereals during the grain-filling period, single grain weight is reduced (Chowdhury and Wardlaw, 1978). Exposure of the ears alone, even for brief periods, is sufficient to elicit this response in wheat. Bhullar and Jenner (1983) reported that briefly elevating temperature of wheat ears reduced total grain weight as a result of a reduction in individual grain weight and a small reduction (2.6-12.8%) in grain number. Warming the ears temporarily reduced the amount of sucrose and other soluble sugars in the grain, but not in the rachis or the floral organs. They suggested that the supply of sugars to the grain was depressed by elevated temperature, but the rate of grain-filling was not reduced. This hypothesis was supported by later studies (Bhullar and Jenner, 1986).

Influence of environmental temperature on starch properties

Properties of endosperm starch have been reported to be affected by genetic and environmental factors during the development of the plants. Although environmental effects are not as pronounced as those associated with genetic variations, environmental temperature could affect amylose content and the primary structure of starch granules such as crystalline structure and granular shape. Other physical properties, such as pasting characteristics, were also reported to be sensitive to the environmental temperature under which the starch granule was produced.

Zuber (1965) reviewed studies on the genetic control of starch development including the environmental influence on the amylose content of corn starch. He reviewed years of experimentation on amylose synthesis including location effects. He noted that high temperatures affected high-amylose strains more than low-amylose strains. No correlation was found between temperature and amylose content for ordinary corn. Fergason and Zuber (1962) showed that a high-amylose line grown in a Florida winter nursery had higher amylose contents than the same lines grown in Missouri during the summer. This observation led to an experiment in which strains ranging in amylose content were grown at eight locations in the United State for a 3-year period. A negative correlation was found between amylose content and temperature during the growing season. The highest average amylose content was obtained at a Wisconsin location that had the lowest cumulative degree days, while the lowest amylose content was obtained at a North Carolina location that had the second highest cumulative degree days.

Zuber (1965) also suggested that conditions of environmental stress should be avoided. The influence of environment on amylose synthesis should be considered by those growing high-amylose hybrids for commercial production. Stress conditions that affect amylose synthesis may result from other factors, such as improper balance of soil nutrients and excessive plant populations. More information is needed on the effects of different cultural practices on amylose synthesis.

Non-waxy starch in rice grains of the Japanese cultivars consists of 15-25% amylose and 75-85% amylopectin. Suzuki et al. (1966) grew rice plants (*Oryza sativa L.*, Cultivar: Koshiji-wase) in a greenhouse at 17, 21, 25, and 30°C for 30 days after heading. The lower the temperature, the higher the "blue value" (absorbance at 680 nm of starch-iodine complex). Rice cultivars grown in Hokkaido, the most northern island of Japan, had higher contents of apparent amylose (21.1- 24.5%) than those (17.6-21.6%) grown in other regions (Inatsu et al., 1974). Differences in amylose concentration depended mainly on rice cultivar. However, this study suggested that cold weather resulted in higher amylose contents in the same rice cultivar. Temperature during rice grain ripening is suggested as the dominant environmental factor affecting amylose content (Gomez, 1979).

Asaoka et al. (1984) grew rice plants, Nippongare, Koshihikari, and Hokuriku cultivars, in a greenhouse at 25 and 30°C after heading. Rice plants grown at 30°C had decreased amylose contents compared with those grown at 25°C. Amylose and amylopectin were affected most by environmental temperature at 5 to 15 days after anthesis, *i.e.*, at an early stage of the grain filling period when endosperm starch

accumulation is most active. Moreover, higher environmental temperature may have increased the amount of long B chains of amylopectin and decreased the short chains of amylopectin in rice endosperm.

To confirm the effect of the environmental temperature on the fine structure of amylopectin, further investigation was undertaken by Asaoka et al. (1985) using waxy and non-waxy near-isogeneic lines of a Japonica rice cultivar, Taichung 65. Non-waxy rice plants grown at 30°C, compared to those grown at 25°C, had decreased amylose contents. Waxy rice had increased amount of long B chains of amylopectin with decreased short B chains and slightly decreased A chains.

Temperature effect on branching enzyme action and amylose conformation

The branching enzyme, Q-enzyme, is required for the synthesis of the α-D-(1→6) branch linkages in amylopectin (Lavintman, 1966; Manners, et al., 1968). Whelan (1971) showed that this branching enzyme catalyzed the transfer of one amylose chain to another chain of average chain lengths (CL) of 48 and 260. The effect of amylose on different chain lengths was also studied (Borovsky and Whelan, 1972). It was shown that only amylose chains of CL > 50 act efficiently as donors or acceptors of the transferred segments. The minimum CL of an amylose for Q-enzyme substrate was 30-40 (Borovsky et al., 1975). The requirement of Q-enzyme for amylose for CL > 50 may be related to the tendency of amylose of this length to form tertiary structures, double helices (Kainuma and French, 1972; Borovsky and Whelan, 1972; Borovsky et al., 1975; Borovsky et al., 1976). The studies of Borovsky and Smith (1974) showed

that the Q-enzyme could react with amylose of smaller chain length at 4°C compared with amylose at 35°C. They also showed that the difference in the branching patterns developed at 4 and 35°C was not caused by differences in temperature coefficients of the Q-enzyme and the potato phosphorylase. These studies suggest that the large proportion of short chains found in native amylopectin may represent residual chains rather than chain segments that have been transferred by Q-enzyme.

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SEPARATION AND QUANTIFICATION OF MALTO-OLIGOSACCHARIDES BY HIGH-PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY WITH PULSED AMPEROMETRIC DETECTION[1]

A paper to be submitted to Carbohydrate Research

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Abstract

High-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) was used to separate and quantify malto-oligosaccharides. Alkaline eluent, 100 mM to 500 mM sodium hydroxide solution, was used for direct detection of carbohydrates on a set of gold working and calomel reference electrodes. Chromatographic capacity factors, k', of malto-oligosaccharides

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[1] Journal Paper No. ####### of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project No. 3258

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increased as molecular size increased. Sodium hydroxide alone was not adequate for an optimal malto-oligosaccharide separation. Sodium acetate, nitrate and sulfate were tested as pushing agents. The salts were compatible with the system, increased the separation selectivity and enhanced the detector response of PAD. The ability of pushing agents to decrease k' followed the order of sulfate > nitrate > acetate > hydroxide. After being eluted with pushing agents, the column regeneration time of CarboPac PA1 was 180, 240 and 360 minutes for acetate, nitrate and sulfate, respectively. A procedure to shorten the regeneration time for malto-oligosaccharide separation was demonstrated in this study. Sodium azide, as a preservative, did not affect the separation and quantification of the analysis. Sodium nitrate was the best pushing agent for the homologous malto-oligosaccharide separation.

Introduction

Carbohydrates, being weakly acidic, can be adsorbed as anions[1, 2] and separated by anion-exchange columns in alkaline pH conditions. Alkaline conditions are, therefore, appropriate for the direct detection of all carbohydrates by using a pulsed amperometric detector (PAD) with an electrode of noble metal[3-5]. PAD, the detection unit, was invented by Hughes et al. for detecting alcohols[6], sugars and sugar alcohols[3] with 100 times more sensitivity than conventional refractive index detectors[4]. PAD utilizes triple-step potential-time waveforms, detection, oxidation and reduction potential. Carbohydrates were oxidized on the PAD electrode surface at the detection

potential in the waveform. Following the detection step, the amperometric detector alternated anodic and cathodic polarizations to clean and reactivate the electrode surface[7].

Rocklin and Pohl[8] introduced high-performance anion-exchange chromatography (HPAEC) with PAD for carbohydrate analysis in 1983. HPAEC with PAD is a powerful separation and analysis tool for carbohydrate chemistry research[9]. Sugar alcohols[10-14], aminosaccharides[13, 14], acidic[13, 15] and neutral monosaccharides[10-14], oligosaccharides[10, 14, 16-19] and polysaccharides[20-21], without any derivatization, have been easily separated by using HPAEC with alkaline mobile phases and directly detected by the highly sensitive and selective PAD.

The chromatographic capacity factor value, k', of carbohydrates increases as molecular size increases and pKa decreases[12]. In general, the affinity of carbohydrates to an anion resin follows the order of sugar alcohols < monosaccharides < oligosaccharides < polysaccharides[8]. A wide range of oligo- and polysaccharides[22-24] (acidic[25-27], glycoconjugate[14, 28], linear[18, 29], branched[29] and cyclic[30]) have been effectively separated with this system. With the HPAEC-PAD system, a homologous series of oligo- and polysaccharide mixtures with different degrees of polymerization (DP), up to 50 or higher[27, 31, 32], and oligosaccharide isomers[33, 34] also have been successfully separated.

For improving carbohydrate separation, pH gradient or pushing agent gradient was used with the HPAEC-PAD system. Pushing agents, non-oxidizable anion salts, were used to change the ionic strength of the eluents and thus optimize retention time

and resolution of large molecular carbohydrates in the anion-exchange column. Sodium hydroxide, acetate, carbonate, nitrate and sulfate were evaluated as pushing agents by Rocklin and Pohl[8]. Acetate was preferred for oligosaccharide separation, because its anion exchange resin affinity is similar to that of hydroxide. A sodium hydroxide gradient[35] and a sodium acetate gradient[8, 20, 21, 30] were used in high pH (12-14) eluents for sugar alcohols, and neutral and basic carbohydrates. Hotchkiss and Hicks[27] used a potassium oxalate gradient in low pH (6.0) eluents for acidic carbohydrate analysis. The observed disadvantages of all of the mentioned procedures include baseline shift[7, 27, 36] caused by acetate or oxalate concentration gradient and different detector responses for different carbohydrates[12, 21, 29, 31].

The purpose of this work was to use linear malto-oligosaccharides to investigate suitable pushing agents and to study the effects of those pushing agents on the detector response.

Experimental

Materials. Glucose (Fisher Scientific, Springfield, NJ), maltose, maltotriose, maltotetraose, maltohexaose, maltoheptaose (Aldrich Chemical Co., Milwaukee, WI), and maltopentaose (Hayashibara Shoii Inc., Okayama, Japan) were used without further purification. Sodium acetate, sodium nitrate and sodium sulfate and 50% (weight percentage) sodium hydroxide stock solution (Fisher Scientific, Pittsburgh, PA), and sodium azide (Mallinckrodt Chemical Works, St. Louis, MO.) were ACS certified grade

chemicals. Triple-distilled water was purified further in a Millipore Milli-Q system (Millipore Corp., Bedford, MA). All mobile phases containing sodium hydroxide were diluted from a 50% sodium hydroxide stock solution, filtered before use (0.45 μm Nylon-66 filter, Rainin Corp., Woburn, MA) and degassed under vacuum.

Chromatographic apparatus. Separations were performed by using a CarboPac PA1 column (250 mm x 4 mm i.d.), a Dionex pulsed amperometric detector with a gold working electrode (Dionex Corp., Sunnyvale, CA) and saturated calomel reference electrode (Fisher Scientific, Springfield, NJ), and a Dionex analytical pump with a 0.8 ml/min flow rate. Pulse potentials (volt) and durations (time, millisecond) used for this work on the PAD were: E_1 = 0.05 V, t_1 = 480 ms, E_2 = 0.7 V, t_2 = 120 ms, and E_3 = -0.7 V, t_3 = 360 ms. The response time of the detector was set for 3 sec. Data were collected with a strip-chart recorder or an integrator (Model 427, Beckman Instrument Inc., Berkeley, CA).

Results and Discussion

The effects of sodium hydroxide concentration on chromatographic capacity factors, k', of malto-oligosaccharides are shown in Figure 1a. The k' of malto-oligosaccharides increased as glucose unit numbers increased, confirming the results of Koizumi et al.[31] With the 100 mM sodium hydroxide solution eluent, the k' of maltotetraose was 120, which was too large for practical analysis. The relationship

between increased k' and increased degree of polymerization (DP) is attributed to the increased number of ionized hydroxyl groups in the alkaline solution, which produced higher molecular adsorption onto the anion exchange resin. An increased concentration of sodium hydroxide in the eluent decreased the k' but resulted in a noisy baseline. At concentrations below 500 mM, the retention of maltoheptaose was too long to be practical, and the peaks were too broad to be accurately integrated. The results indicated that an eluent of at least 500 mM of sodium hydroxide was needed when applied alone to reduce the k' of maltoheptaose to a reasonable range for analysis, improving peak integration. At 500 mM of sodium hydroxide or above, the detector noise was too high to perform an accurate and reliable analysis; additionally, column pressure increased. Therefore, the addition of a pushing agent to the eluent is essential for accurate analysis.

Sodium salts of acetate, nitrate, and sulfate were used to increase the ionic strength of the eluent and to function as pushing agents. A baseline separation of a mixture of glucose to maltoheptaose could be achieved with a k' value less than 10 by adjusting the concentration of the selected pushing agents. The pushing agents effectively decreased the k' without increasing baseline noise. The effects of the pushing agents on malto-oligosaccharide k' are shown in Figures 1b-d. On an isocratic system, the optimal pushing agent concentrations for malto-oligosaccharide separation on a CarboPac PA1 column with a 100 mM sodium hydroxide basis were 200 mM, 30 mM and 15 mM for acetate, nitrate and sulfate, respectively. The differences in the optimum concentrations of the pushing agent was a result of their affinity for the anion

exchange resin. A pushing agent with higher anion exchange resin affinity can be effective at a lower concentration. The ability of pushing agents to decrease k' followed the order of sulfate > nitrate > acetate > hydroxide. The system performed a precise analysis and the deviation of k' of malto-oligosaccharides in all analytical conditions was 1% or less. Sodium azide, a preservative, was well separated from malto-saccharides and did not affect the separation and quantification of malto-oligosaccharides (data not shown).

For comparing concentration effects of the pushing agents on malto-oligosaccharide detector responses, the response of maltose in 100 mM sodium hydroxide was assigned a value of 100 and a relative detector response for each sugar was used in all of the analysis. Detector responses of malto-oligosaccharides were affected by concentrations of sodium hydroxide and pushing agents (Figure 2). Glucose had a distinctly higher detector response than other malto-oligosaccharides in all eluents tested. The high detector response of glucose may be due to its small molecular size and a high proportion of the latent aldehyde group that can be promptly oxidized to acid. The detector responses of malto-oligosaccharides decreased as molecular size decreased. The reason for this detector response decrease is not well understood. Detector responses of the malto-oligosaccharides at different sodium hydroxide concentrations and with pushing agents differed. The detector responses of malto-oligosaccharides at different sodium hydroxide concentrations are shown in Figure 2a. The plot of $\log_{10}$ (detector response) versus malo-oligosaccharide degree of polymerization yielded a line. The detector-response slope was enhanced by sodium

hydroxide concentration. As the concentration increased, the slope of the line decreased (Figure 2a). This effect was apparent on detector responses for maltotriose, maltotetraose and maltopentaose. The detector responses for these malto-oligosaccharides in 400 mM and 500 mM sodium hydroxide were significantly larger than the responses in 100 mM or 200 mM. The deviation of detector response in this analysis using sodium hydroxide alone as the eluent was high because of the difficulty of peak integration. Two reasons for the difficulty in obtaining accurate detector responses under these operating conditions were: first, the higher concentrations of sodium hydroxide resulted in higher background noise; and second, peaks with high k' were broadened. In contrast, the detector response slopes of malto-oligosaccharides eluted by mixtures of 100 mM sodium hydroxide and different pushing agent concentrations (Figure 2b-d) showed no significant differences, except the mixtures with 50 mM sodium acetate and 20 mM sodium sulfate. The 50 mM sodium acetate mixture did not elute maltohexaose and maltoheptaose within 90 min. Conversely, the sodium hydroxide with 20 mM sodium sulfate mixture was so powerful that all the malto-oligosaccharides were eluted within 4 min; this produced partially overlapped glucose, maltose, and maltotriose peaks.

To quantify the malto-oligosaccharide analysis, reproducible linear relationships between sugar concentration and detector response are essential. Results of six different concentrations of malto-oligosaccharides subjected to HPAEC-PAD analysis and eluted by 500 mM sodium hydroxide showed good linear PAD response, although the detector response of each sugar was different. The linear relationships between concentrations

and detector responses of maltose, maltotriose, maltopentaose and maltoheptaose in 500 mM sodium hydroxide are shown in Figure 3. The detector responses of malto-oligosaccharides eluted by 100 mM sodium hydroxide and pushing agents (acetate, nitrate and sulfate) were similar, but greater than those eluted by 500 mM sodium hydroxide alone (Figure 4). Correlation coefficients of detector response and malto-oligosaccharide concentrations eluted by different eluents are shown in Table 1. Standard deviations of malto-oligosaccharide detector responses showed that acetate and nitrate added to eluent generated more precise measurements than sulfate and sodium hydroxide alone (100 mM and 500 mM) (Figure 5).

Based on the ability to control k', detector response, linearity and reproducibility, acetate and nitrate are good pushing agents. Sodium nitrate is a neutral salt with a higher solubility than sodium acetate (88g and 46.5g, in 100g water at 20°C, respectively)[37]. This property makes sodium nitrate a versatile pushing agent that also can be used in a neutral eluent. Relative viscosities for 1% solutions of sodium hydroxide, acetate, nitrate and sulfate at 20°C are 1.052, 1.038, 1.005 and 1.024, respectively[38]. The lower column pressure, resulting from the lower viscosity of sodium nitrate solution, is another of its advantages as a pushing agent.

When the anion exchange resin is at an alkaline pH, the majority of anion exchange groups are bound to hydroxide ions. When a pushing agent was used, following each separation, the hydroxide ions are replaced by anions of the pushing agent. Before loading the next sample, the resin must be regenerated to the OH-form. Glucose was used to evaluate the CarboPac PA1 column regeneration time. The times

required to regenerate the column for different pushing agents were different. The regeneration times for eluent mixtures of 100 mM sodium hydroxide with 400 mM acetate, 60 mM nitrate and 30 mM sulfate, returning to 100 mM hydroxide base eluent, were 180, 240 and 360 min, respectively (Figure 6). Regeneration time was related to the affinity of the pushing agent. The time periods required for column regeneration were too long for practical operation.

The column regeneration time can be shortened by using a low concentration pushing agent and sodium hydroxide mixture as a base eluent (*e.g.*, Figure 7). Maltose was used to evaluate the column regeneration time, from working eluent (high pushing agent concentration) to the base eluent (low pushing agent concentration), in which glucose retention time was too short to precisely evaluate the time difference. For malto-oligosaccharide separation, the column can be regenerated within 30 min regardless of the pushing agent used.

Under alkaline conditions on HPAEC, the malto-oligosaccharide column capacity factor increased as degree of polymerization increased. Non-oxidizable anion salts as pushing agents were essential for an accurate analysis of malto-oligosaccharides. The abilities of anions to adjust the chromatographic capacity factors of malto-oligosaccharides followed the order of sulfate > nitrate > acetate > hydroxide. On a weight concentration basis using PAD, the detector response of malto-oligosaccharides decreased as degree of polymerization increased. The use of pushing agents also enhanced the detector response of malto-oligosaccharides. Acetate and nitrate both performed as good pushing agents, with the latter having a higher ability to control the

malto-oligosaccharide chromatographic capacity factor. Sodium nitrate was found to be a versatile pushing agent in a HPAEC, which can be used at alkaline and neutral pH for separating and elucidating basic, acidic and neutral polysaccharide molecular structure.

Acknowledgments

The authors thank Dr. Dennis C. Johnson at Iowa State University for allowing us to use his equipment and Barbara Anderson for technical assistance. Financial support for this project was provided by ICI Seeds Company, Slater, IA.

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Table 1. The Correlation Coefficients[1] of Detector Response and Weight Concentration of Malto-oligosaccharides.

Malto-saccharides	500 mM NaOH	200 mM CH_3COONa + 100 mM NaOH	30 mM $NaNO_3$ + 100 mM NaOH	15 mM Na_2SO_4 + 100 mM NaOH
Glucose	0.973	0.978	0.943	0.907
Maltose	0.995	0.999	0.994	0.989
Mlatotriose	0.989	0.998	0.992	0.991
Maltotetraose	0.983	0.998	0.995	0.993
Maltopentaose	0.967	0.988	0.974	0.959
Malotohexaose	0.930	0.998	0.988	0.977
Maltoheptaose	0.934	0.998	0.994	0.983

[1] Results from triplicate samples at six different concentrations.

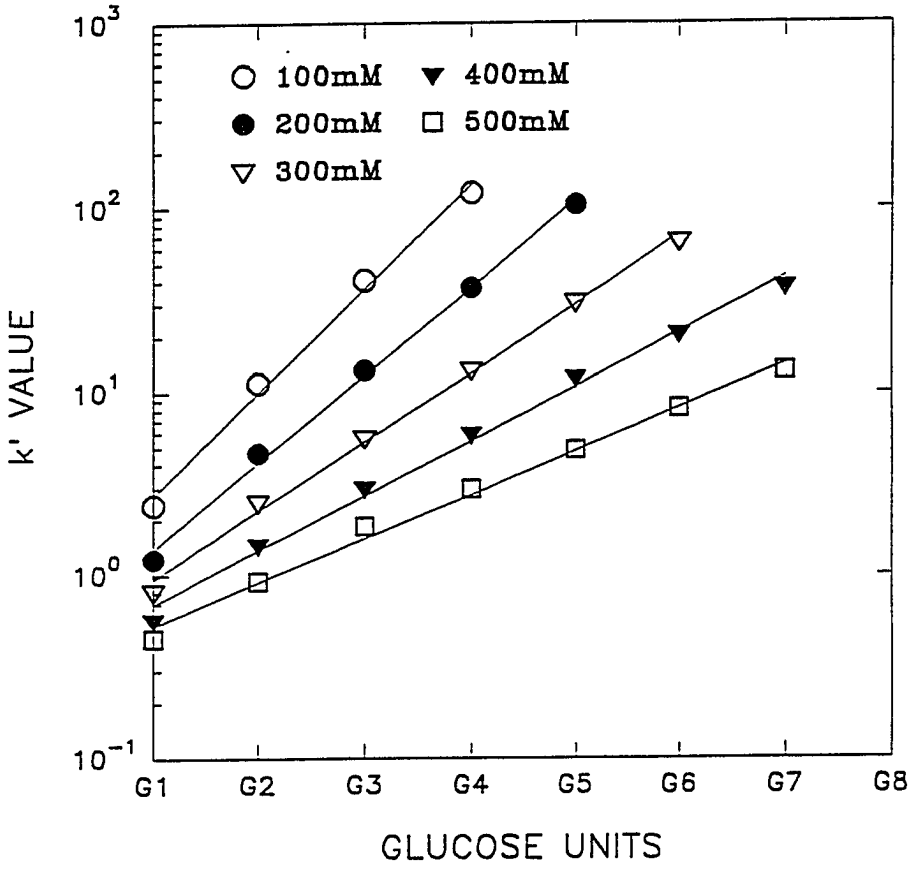

Figure 1a. The effect of concentration of sodium hydroxide as a pushing agent on chromatographic capacity factors for malto-oligosaccharide on CarboPac PA1 column. The retention time of water used as an unretained component for calculating capacity factor was 1.4 min.

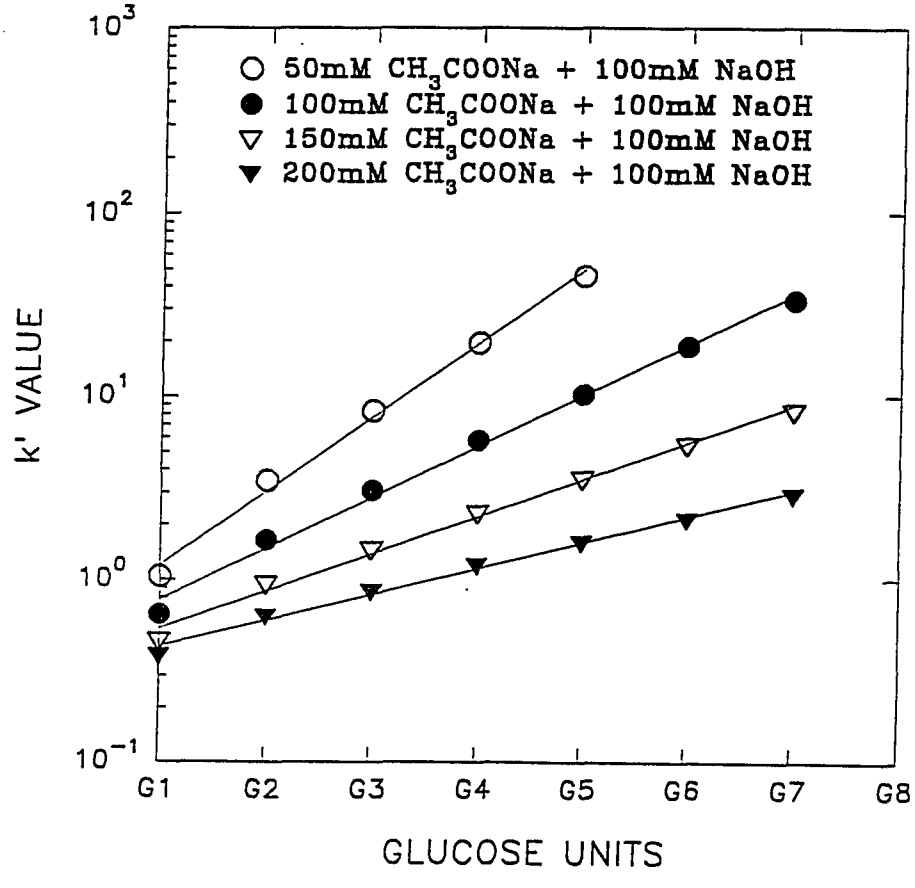

Figure 1b. The effect of concentration of sodium acetate as a pushing agent on chromatographic capacity factors for malto-oligosaccharide on CarboPac PA1 column. The retention time of water used as an unretained component for calculating capacity factor was 1.4 min.

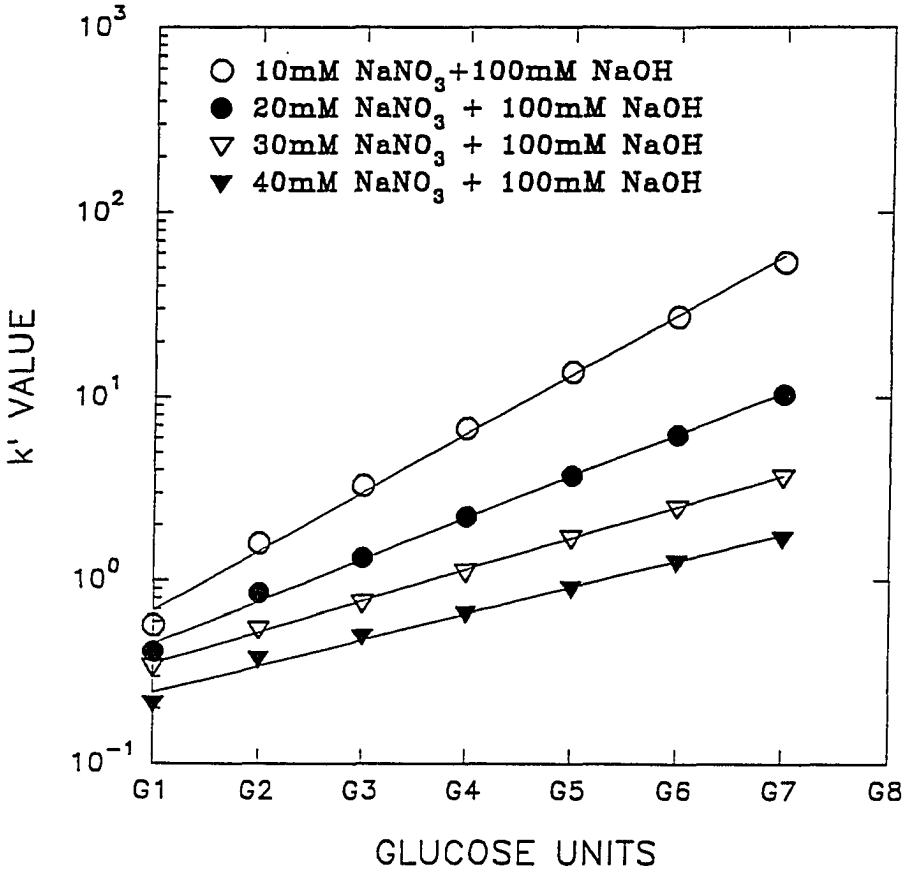

Figure 1c. The effect of concentration of sodium nitrate as a pushing agent on chromatographic capacity factors for malto-oligosaccharide on CarboPac PA1 column. The retention time of water used as an unretained component for calculating capacity factor was 1.4 min.

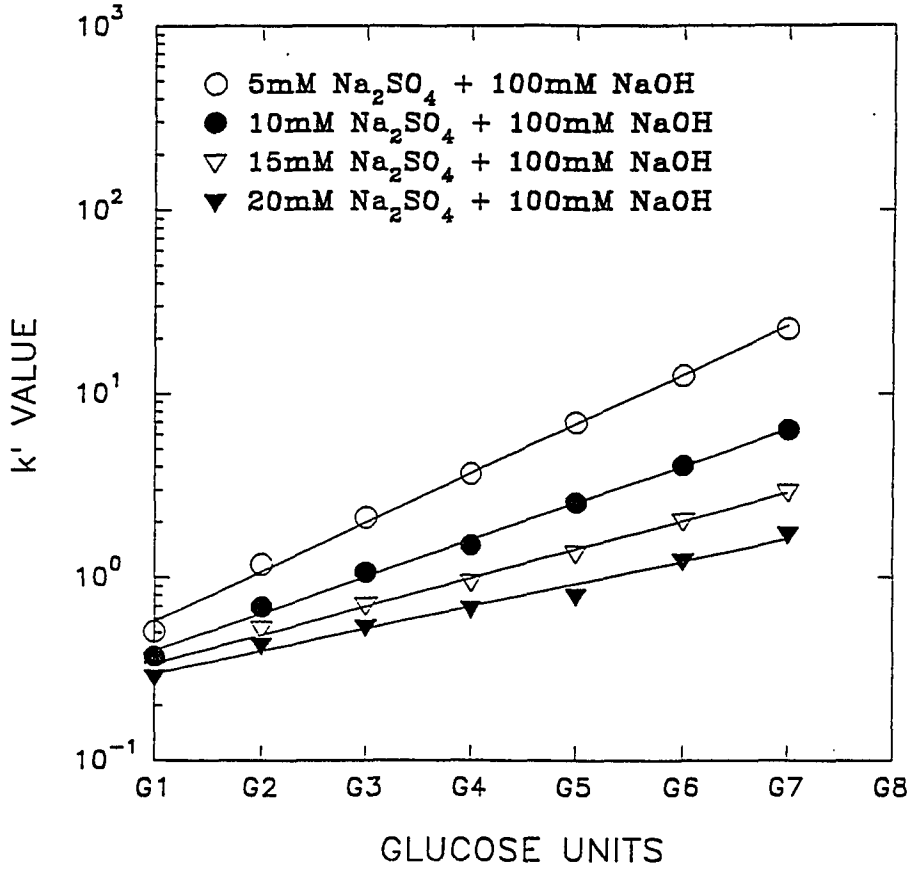

Figure 1d. The effect of concentration of sodium sulfate as a pushing agent on chromatographic capacity factors for malto-oligosaccharide on CarboPac PA1 column. The retention time of water used as an unretained component for calculating capacity factor was 1.4 min.

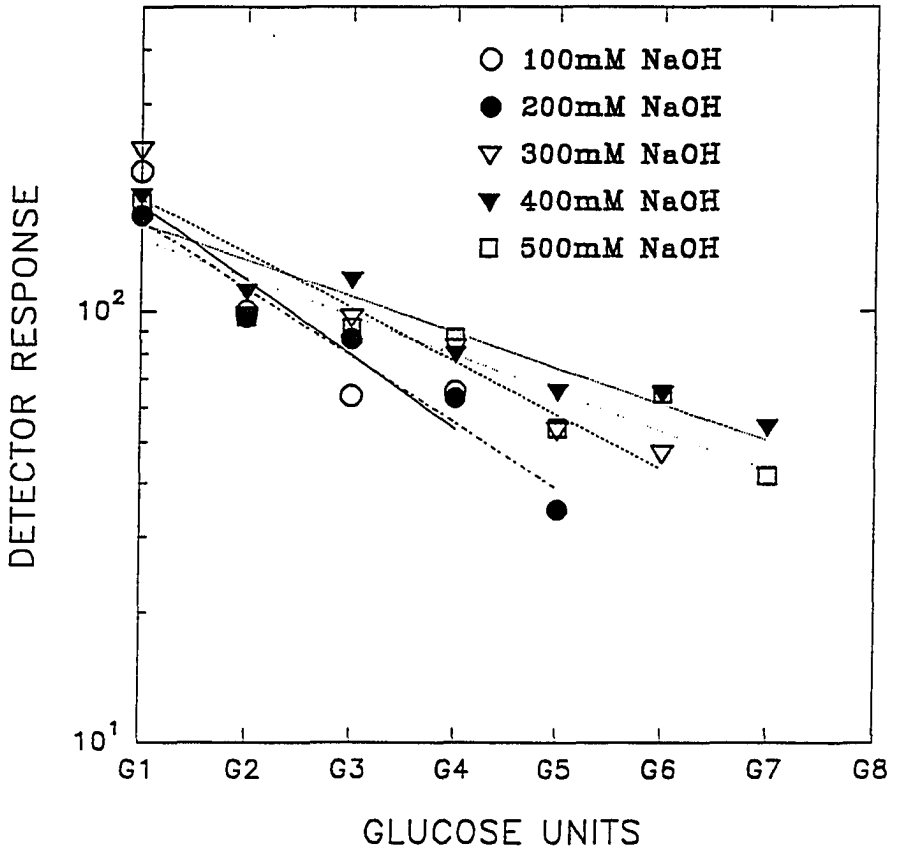

Figure 2a. The effect of concentration of sodium hydroxide as a pushing agent on PAD detector responses of malto-oligosaccharide. The detector response of maltose in 100 mM sodium hydroxide was assigned as 100.

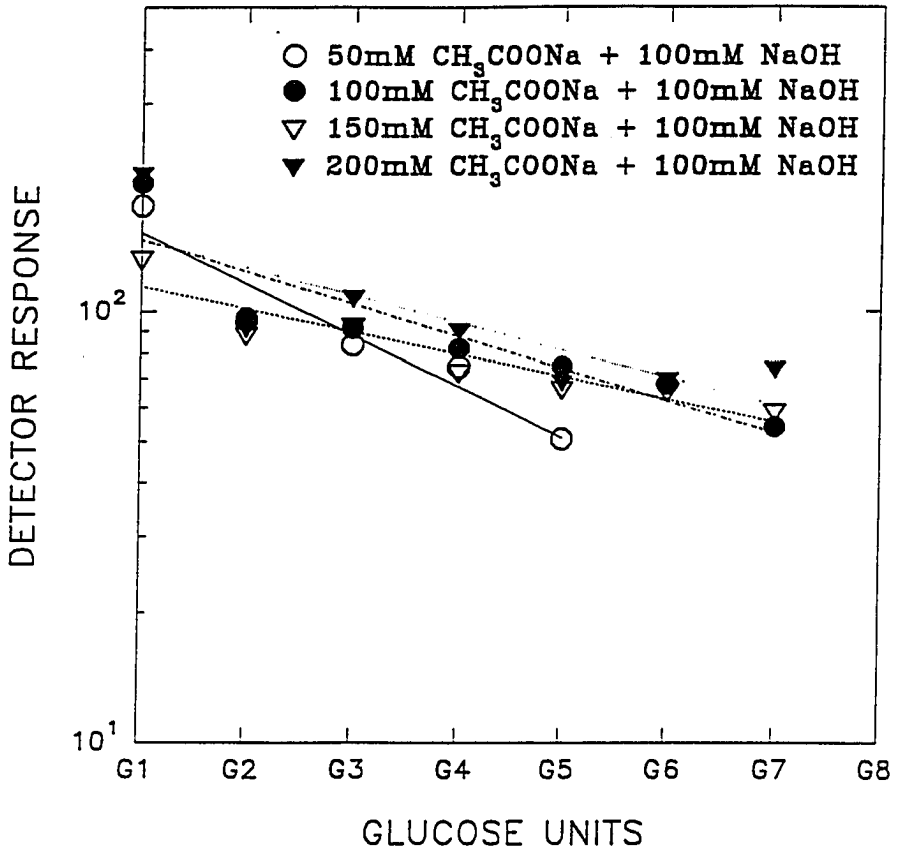

Figure 2b. The effect of concentration of sodium acetate as a pushing agent on PAD detector responses of malto-oligosaccharide. The detector response of maltose in 100 mM sodium hydroxide was assigned as 100.

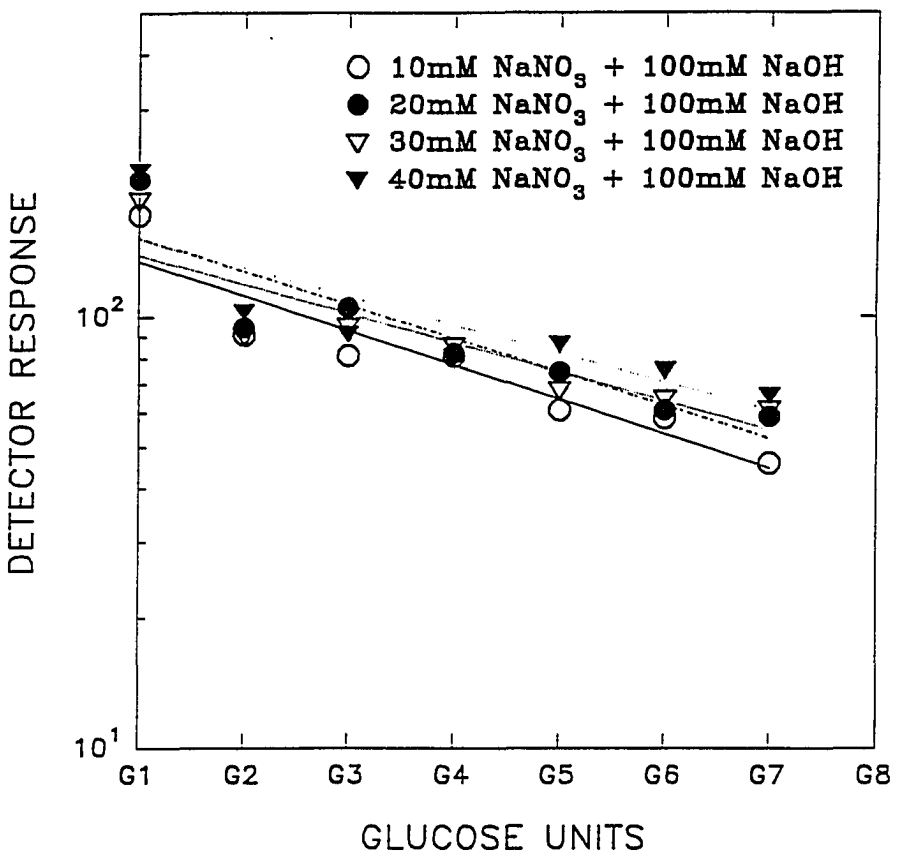

Figure 2c. The effect of concentration of sodium nitrate as a pushing agent on PAD detector responses of malto-oligosaccharide. The detector response of maltose in 100 mM sodium hydroxide was assigned as 100.

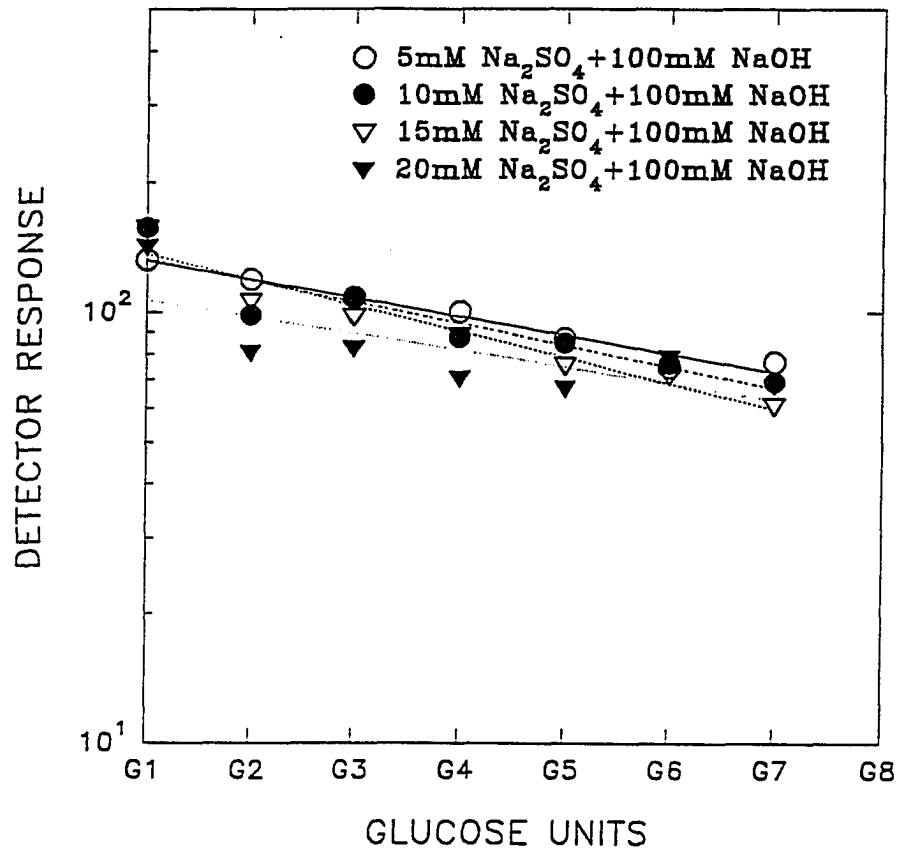

Figure 2d. The effect of concentration of sodium sulfate as a pushing agent on PAD detector responses of malto-oligosaccharide. The detector response of maltose in 100 mM sodium hydroxide was assigned as 100.

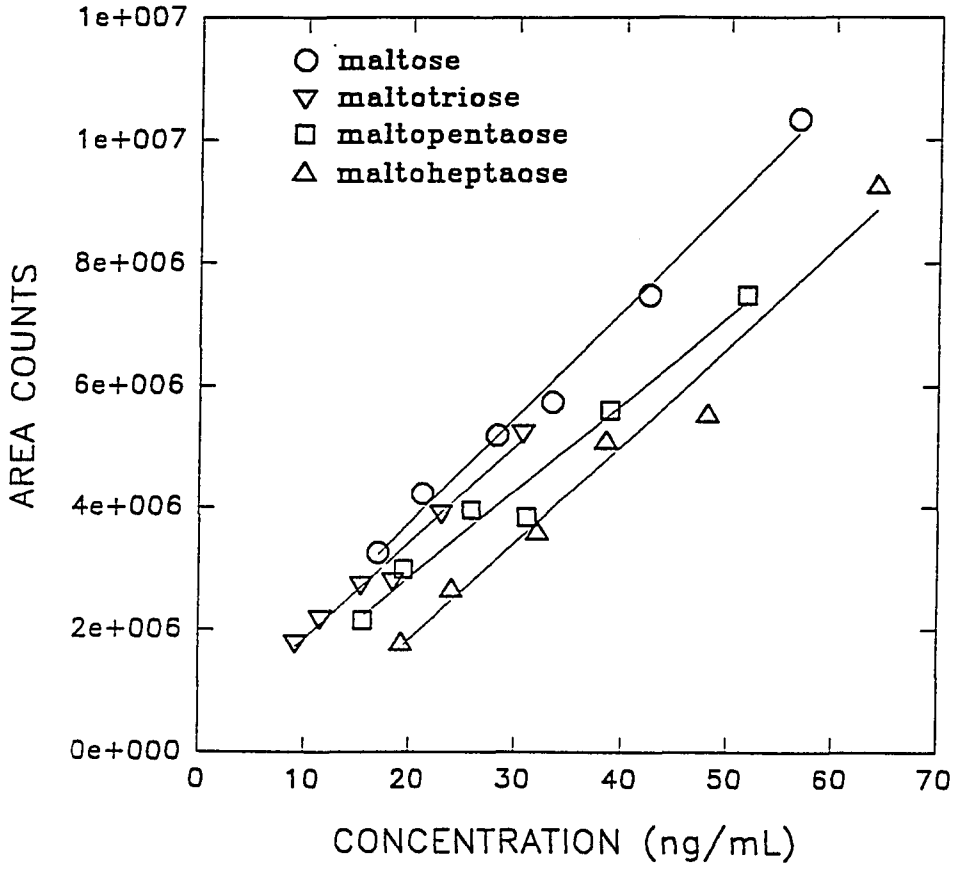

Figure 3. The relationship between concentration of malto-oligosaccharide and PAD detector response in 500 mM NaOH eluent. (data are the average of triplicate samples.)

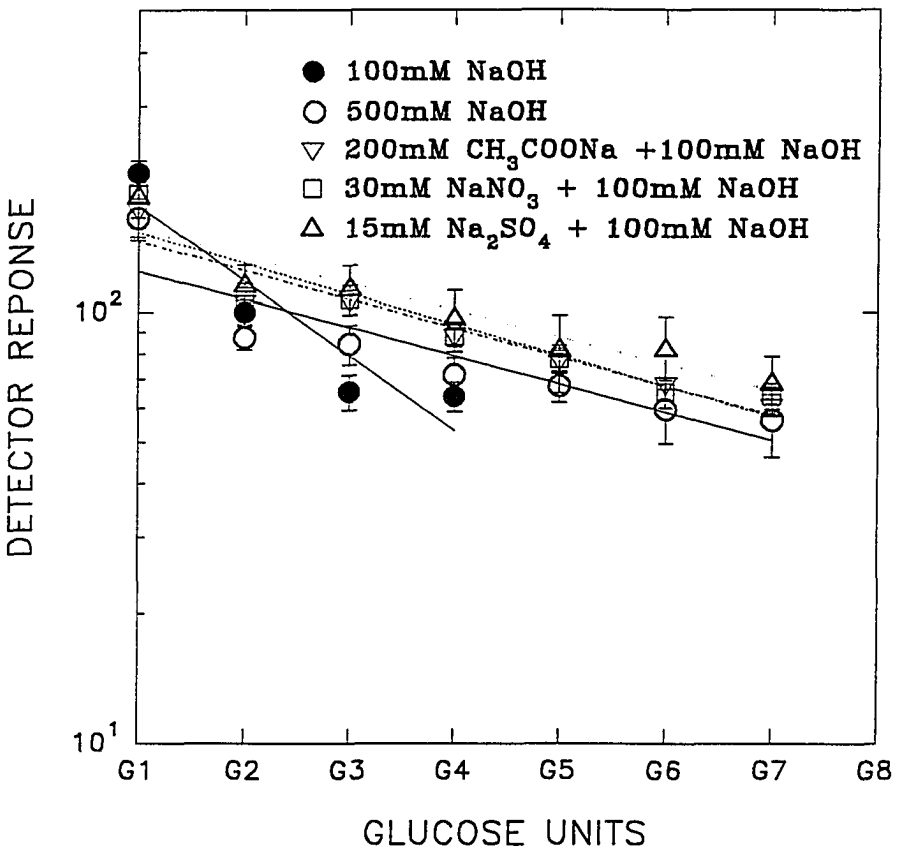

Figure 4. Different pushing agents on PAD detector responses of malto-oligosaccharide. (the data are the average of six different concentrations of triplicate samples.)

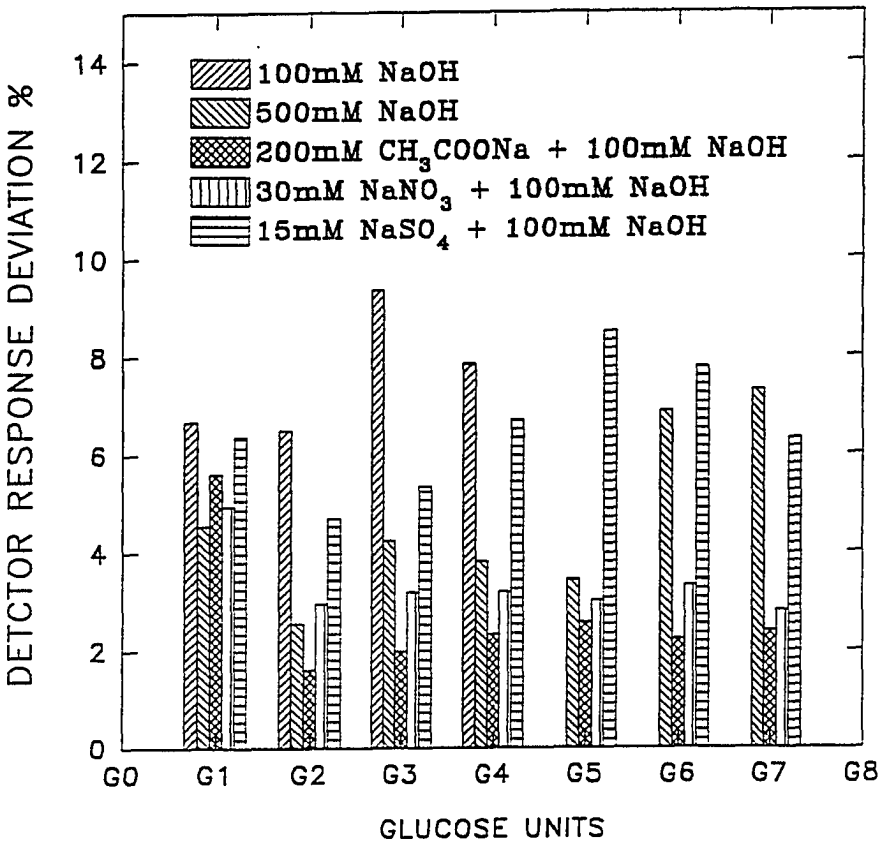

Figure 5. The percent deviation of the detector responses for malto-oligosaccharides on PAD. The deviation values were calculated from triplicate samples at six different concentrations.

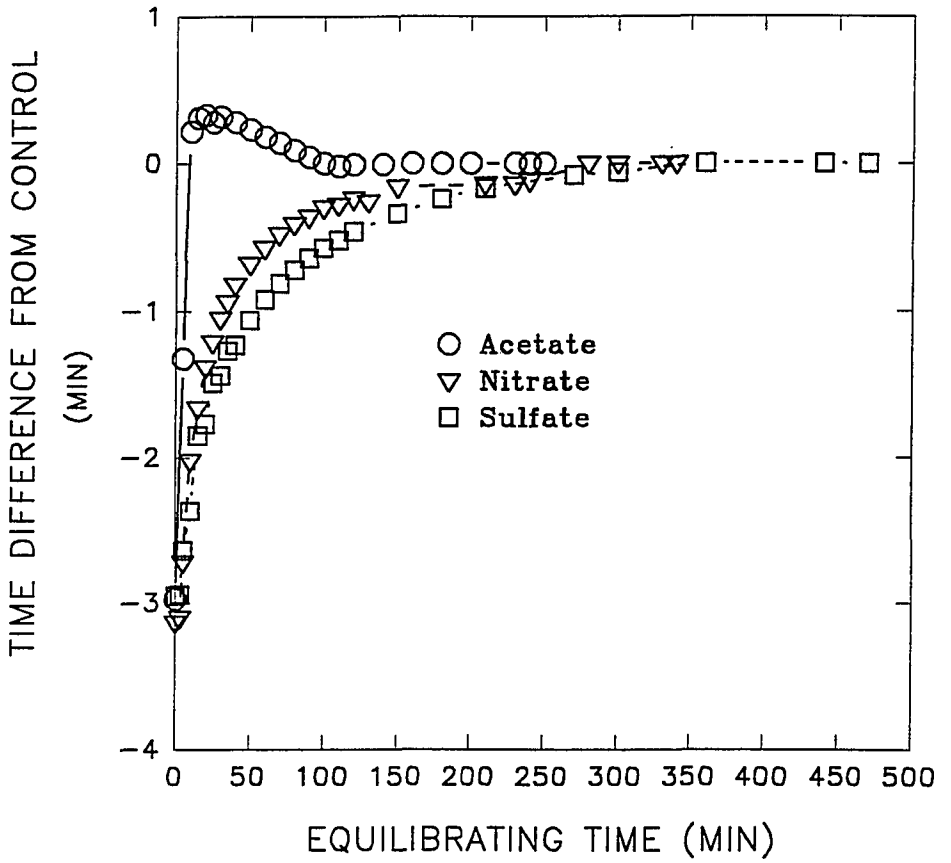

Figure 6. The regeneration time of CarboPac PA1 column. Testing conditions: Switching eluent from 400 mM sodium acetate, 60 mM sodium nitrate and 30 mM sodium sulfate with 100 mM sodium hydroxide to 100 mM sodium hydroxide alone. Retention time of glucose in 100 mM sodium hydroxide was used as a control. Data are the average of triplicate samples.

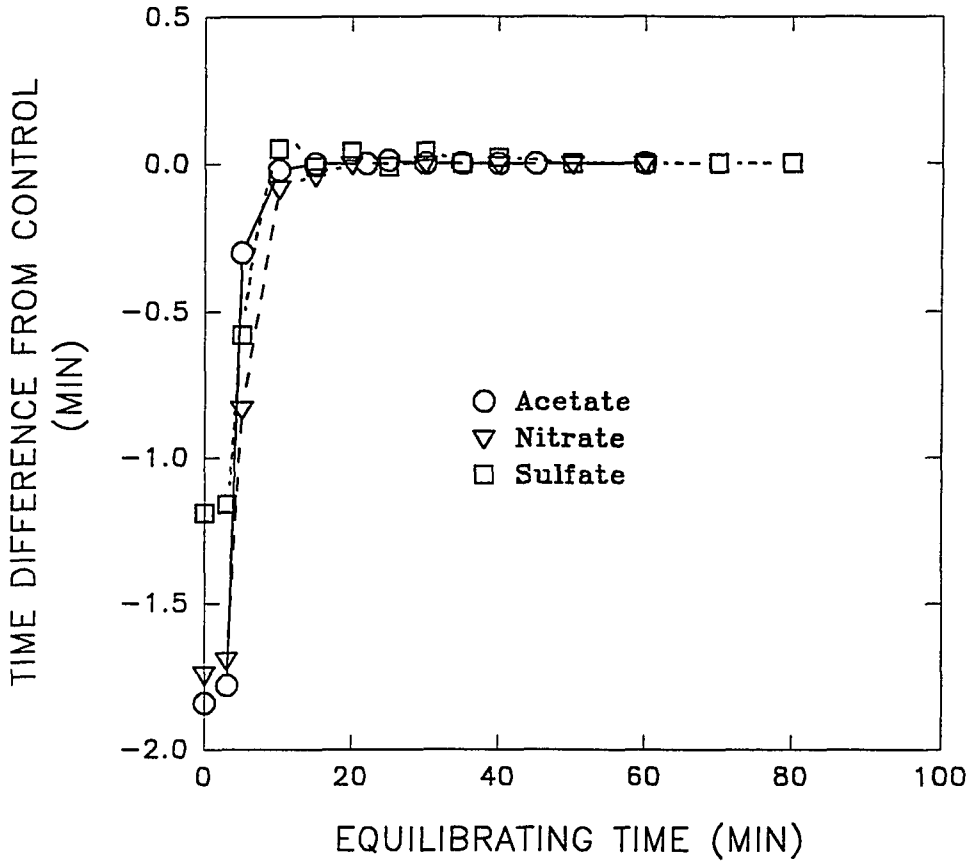

Figure 7. The regeneration time of CarboPac PA1 column from high concentration to low concentration of pushing agents. Testing conditions: Sodium acetate from 400 to 100 mM, sodium nitrate from 60 to 10 mM and sodium sulfate from 30 to 5 mM with 100 mM sodium hydroxide as base. Retention times of maltose in low concentrations of pushing agents were used as controls. Data are the average of triplicate samples.

EFFECTS OF EAR DEVELOPMENTAL TEMPERATURE ON FINE STRUCTURE OF MAIZE STARCH[1]

A paper submitted to Carbohydrate Research

Ting-jang Lu[2], Jay-lin Jane[2,3], Peter L. Keeling[4], George W. Singletary[4]

Abstract

Growing temperature is known to affect the grain yield and quality of maize. Two genetically unrelated normal dent maize inbreds, ICI63 and ICI92, with different heterotic backgrounds were grown in a greenhouse with the ears wrapped in temperature control devices set at 25 and 35°C during the grain-filling period. Grain yield, kernel weight, and kernel density were less for ears at 35°C than those at 25°C. The extent of the loss, however, varied with the variety: 13.1 and 37.9% kernel weight loss and 8.47

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[1] Journal Paper No. J-16320 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project No. 3258

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and 10.08% density loss for ICI63 and ICI92, respectively. The starch granule shape of ICI63 became more oval-shaped, but there was no shape change for ICI92. As growing temperature increased, starch granule size decreased, and gelatinization onset temperature increased. With increased developmental temperature, amylose content, determined by iodine affinity, decreased 2.39% for ICI63 and 2.20% for ICI92; amylose molecular size of both varieties also decreased. When the ear developed at 35°C, size-exclusion chromatography and high- performance anion exchange chromatography revealed an increased medium branch-chain fraction and decreased long and short branch-chain fractions for ICI63 amylopectin, whereas ICI92 amylopectin possessed increased long and medium branch-chain fractions and decreased short branch-chain fraction.

Introduction

Temperature is one environmental variable that cannot be manipulated in the field, and crops are often selected for a region on the basis of regional temperature. Temperature fluctuations affect growth and yield of grains: wheat[1-8], rice[1, 8-10], sorghum[1], and maize[11-15] and tubers: cassava[16] and potato[17-19]. Changes in growth temperature result in different whole-plant dry matter[15], mature grain weight[8], and starch properties[9-11, 20]. Brown[12] reviewed maize environmental temperature responses and identified four major effects: 1) development rate was not a direct function of temperature; 2) developmental response to temperature was not the same in all developmental

subperiods nor was it the same for all cultivars; 3) other environmental variables needed to be considered in some developmental subperiods; and 4) temperature fluctuations, particularly diurnal range differences might affect development rate, and the timing of the fluctuations might be very important.

Starch biosynthesis is directly affected by environmental temperature[21-24]. Temperatures higher than the optimum reduce the starch deposition rate in cereal grains[1, 6, 7] and potato tubers[18, 19] and also shorten the duration of wheat and rice grain filling[8, 25]. Because temperature changes affect starch biosynthesis, the structures of starch from different developmental temperatures differ. High temperature significantly decreases the amylose contents in rice[9] and high-amylose maize starch[11] and changes the fine structure of rice amylopectin[10]. Different developmental temperatures change starch structures and result in property differences. This variability in starch quality necessitates continuous adjustments of many industrial processing parameters and results in quality control problems for various products.

The studies mentioned, except those works of Bhullar and Jenner [6, 7] on wheat, involved temperature and other environmental variable effects on whole plants. The objective of this work was to reveal, by minimizing other environmental variables, effects of developmental temperature of maize ear on maize grain quality and on starch fine structure.

Experimental

Materials. The following materials were purchased and used without modification: Spectra/Mesh macroporous 53 μm nylon filters (Spectrum, Los Angeles, CA); amyloglucosidase (EC 3.2.1.3) from *Rhizopus* mold (Sigma Chemical CO., St. Louis, MO); Glucose Diagnostic Kit 115-A (Sigma Chemical CO., St. Louis, MO); Isoamylase (EC 3.2.1.68) from *Pseudomonas amyloderamosa* ATCC 21262 (Hayashibara Biochemical Laboratories, Inc., Okayama, Japan); Sepharose CL-2B gel (Pharmacia Inc., Piscataway, NJ); and Bio-Gel P-6 gel (Bio-Rad Laboratories, Hercules, CA).

Production of starch at different developmental temperatures. Two genetically unrelated normal dent maize inbreds, ICI63 and ICI93, with different heterotic backgrounds were produced by ICI Seeds, Slater, IA. The maize inbreds are used in commercially available hybrids and are proprietary to ICI Seeds. The maize plants were grown in an environmentally controlled greenhouse with a temperature of 20-25°C and a photoperiod of 15 hr. After 14 days post-pollination, heating mantles were placed on the maize ears and a thermocouple was inserted between the developing kernels and the husk for controlling the ear developmental temperature at 25 and 35°C until maturity, as defined by black layer formation. The 14-day post-pollination waiting-period was to avoid adverse effects on ear development[3,9]. The 25°C sample group was used as the control, because the greatest maize yields are associated with daytime maximums of 24-

30°C[26]. Grain from six ears at 25°C was collected, bulked together, and called the 25°C sample; grain from each of four heated (35°C) ears was maintained separately for kernel dry weight and kernel density determination, then bulked together for other analysis, and called the 35°C sample.

Determination of kernel dry weight and kernel density. Kernel dry weight was measured by using 10 kernels for each sample, after the kernels were dried in a forced-air oven at 80°C for 48 hr. To adjust moisture for kernel density determination, the kernels were equilibrated to approximately 12% moisture in a temperature-humidity controlled incubator at 27°C and 67% relative humidity. Kernel density was measured by using a Multi-Pycnometer Model MVP-1 (Quantachrome Crop., Syosset, NY) with nitrogen gas.

Determination of proximate chemical composition. For determination of proximate chemical composition, maize kernels were ground by using a coffee mill (Model KSM2, Braun, Lynnfield, MA). Kernel moisture was determined by following the modified vacuum-oven method, AACC Approved Methods of Analysis 44-40[27]. Protein content was determined by following the Kjeldahl method, AACC Approved Methods of Analysis 46-12[27], and 6.25 was used as the nitrogen-protein conversion factor. Kernel starch content was measured by following AACC Approved Methods of Analysis 76-11[27] with modifications in which the glucose content of the hydrolysate was analyzed by using a Sigma Diagnostics Kit for Determination of Glucose 115-A[28].

Starch isolation and fractionation. Starch from four samples (two inbreds at two developmental temperatures) was isolated in the laboratory by following the methods of Badenhuizen[29] with minor modifications. The kernels were soaked in a 0.01 M mercuric chloride solution for two days to soften the kernels and to prohibit the activity of kernel amylase. The soaked kernels were blended by using a Hamilton Beach blender, Model 585-1 (Hamilton Beach Inc. Washington, NC) with two parts of 0.01 M mercuric chloride solution for 3 min. The germ and fiber residues were removed by filtering through gauze and a 53-μm nylon Spectra/Mesh macroporous filter. The protein was removed from the starch slurry by using a sodium chloride solution (0.1 M) with saturated toluene. Starch was collected by using a higher relative centrifuge force (3,500 x g, 20 min) than described to prevent loss of small starch granules.

Fractionation of amylose and amylopectin was carried out by following the general procedure of Schoch[30] with slight modifications[31]. The recrystallization procedures for purifying amylopectin and amylose were repeated four times.

Determination of starch granule size. The isolated starch was mounted on a glass microscope slide and viewed with a Zeiss axiophot microscope (Zeiss-Kontron, Thornwood, NY) at 50x magnification (20x by 2.5x optivar). Images of the starch granules were obtained by following the procedure described by Jane et al.[32].

Starch X-ray diffraction pattern. Starch samples were moistened by equilibrating them in a saturated relative humidity chamber for one day at room temperature. Starch

X-ray diffraction was performed on a Siemens D-500 x-ray diffractometer (Siemens, Madison, WI) with copper Kα radiation. The signal of reflection angle, 2θ, from 4 to 40 degrees, was recorded. Other operations followed procedures described elsewhere[32,33].

Determination of starch thermal properties. Starch-water suspension (30%) was sealed in an aluminum pan (Perkin-Elmer, Norwalk, CT) and allowed to equilibrate at room temperature for two hours before each analysis. The gelatinization temperature and enthalpy change of starch were determined by using a differential scanning calorimeter (DSC-7, Perkin-Elmer, Norwalk, CT) following the procedure of Jane et al.[32]. An identical empty aluminum pan was used as the reference.

Determination of iodine affinity of starch components and amylose content. The iodine affinities of amylose, amylopectin, and defatted starch[34] were determined by an automatic potentiometric titrator (702 SM Titrino, Metrohm, Herisau, Switzerland) following the procedure of Schoch[35]. Data were recorded by using Metrodata software (Vesuv 2.0, Metrohm, Herisau, Switzerland) on a personal computer. The amylose content was calculated following the method of Takeda et al.[36]. The measurement deviation of amylose content was calculated from standard deviations of starch, amylopectin, and amylose[37] and statistical comparison was also performed[38]. Apparent amylose content was calculated by dividing the iodine affinity of the starch by 19.0%, the typical value of iodine affinity for purified maize amylose[35].

Starch components profile. The starch solution was prepared by dispersing starch in a DMSO solution, heating the solution in a boiling water bath (96°C) for 30 min, stirring at room temperature overnight, precipitating and washing the starch precipitate with methanol, redissolving in hot water, and filtering through Whatman No. 52 filter paper. Five milliliters of the starch solution containing 15 mg of starch was injected into a liquid chromatograph column packed with Sepharose CL-2B gel (2.6 i.d. X 90 cm). The eluant containing 1 mM NaOH and 25 mM NaCl was applied in an ascending direction with a flow rate of 30 mL/h, and 4.6-mL fractions each were collected and analyzed for total carbohydrate and iodine-staining blue value by following the procedure of Jane and Chen[31].

Molecular structure of amylopectin. The amylopectin was debranched with *Pseudomonas* isoamylase[39]. The debranched sample was filtered through a 0.45-μm nylon syringe filter (Alltech Associates, Deerfield, IL). The branch-chain length distribution profile of amylopectin was determined by using a Bio-Gel P-6 gel permeation chromatograph column (1.5 i.d. x 95 cm). After injection of 2 mL hydrolysate containing 10 mg of debranched amylopectin, samples were eluted with water in a descending direction with a flow rate of 30 mL/h. Fractions (2 mL each) were collected and analyzed for carbohydrate concentration by an anthrone-sulfuric acid method[31,40] and reducing sugar concentration by a modified Park-Johnson method[31,41]. The peak chain length was calculated by dividing the total carbohydrate concentration by reducing sugar concentration.

Chain-length distribution of amylopectin was also performed by using a high-performance anion exchange chromatograph (HPAEC) (Dionex, Sunnyvale, CA) equipped with a DX-300 Gradient Chromatography System, a Pulsed Amperometric Detector (PAD), and a CarboPac PA1 (4 x 250 mm) column and a CarboPac PA guard column (3 x 25 mm) (Dionex). A sodium nitrate gradient was applied as described by Wong and Jane[42].

Statistical analysis. Data were analyzed by using Student's t test in a general linear model (GLM) procedure on a SAS system (release 6.06, SAS Institute, Cary, NC). Means, standard deviations, and significance levels were calculated.

Results and Discussion

To avoid temperature effects on other parts of the plants (e.g., roots and leaves), a heating mantle was used to wrap each individual maize ear to control the developmental temperature of starch. Results showed that the dry weight of kernels developed at 25°C was greater than that at 35°C (Table 1). The effect of temperature on kernel dry weight was more pronounced for ICI92 (37.9% decrease) than for ICI63 (13.1% decrease) when temperature was increased from 25 to 35°C. Additionally, at the 25°C developing temperature, kernel dry weight of ICI92 was 24.9% greater than that of ICI63. At 35°C, however, the weight of ICI63 was only 5.2% greater than ICI92.

Kernels developed at 25°C had greater density than those at 35°C. When temperature increased from 25°to 35°C, ICI92 suffered a greater kernel density loss (10.08%) than did ICI63 (8.47%). This kernel density loss is an important quality concern because neither of the kernels grown at 35°C met the criteria for kernel density for dry milling food-grade maize in which the major grit fraction must be greater than 1.27 g/mL[26].

Table 2 shows proximate chemical compositions of the maize kernels. Moisture contents of these samples were 12 to 13%; the protein contents were about 12%. ICI63 had greater starch contents (ca 70%) than ICI92 (64.1 and 62.0% for 25 and 35°C, respectively). Temperature variation did not significantly affect the proximate chemical compositions of the kernels. From the kernel dry weight and proximate composition data, the total dry matter of the maize kernel decreased as developmental temperature increased. ICI92 suffered more severe dry matter loss than did ICI63.

ICI63 had greater dispersity of granule size distribution than did ICI92 (Table 3). Both ICI63 and ICI92 grown at 35°C had greater populations of small granules. The number average starch granule size of ICI63 decreased from 11.96 to 10.45 μm, whereas the average of ICI92 decreased from 10.78 to 10.33 μm as temperature increased from 25° to 35°C (Table 3). The length/width (L/W) index, the value of maximum diameter divided by minimum diameter, was used as an index of the starch granule shape. L/W values equal 1 for the perfect round shape. The average L/W index of ICI63 increased from 1.19 to 1.24, which indicated more oval-shaped starch granules as temperature increased from 25 to 35°C, but no effect was found on the L/W

index of ICI92. The data support the early observation of Badenhuizen[43] that smaller and more abnormal-shaped starch granules are found in waxy maize starch developed at 30°C incubation than those at 24°C. The results also showed that the impact of developing temperature on starch granule shape varied between different varieties.

X-ray diffractograms of both starch varieties were similar and all showed a typical A pattern (Figure 1). The thermal properties of maize starch, however, varied with the developmental temperature change. ICI63 had a higher gelatinization onset temperature and a narrower range than did ICI93 (Table 4). When the developmental temperature increased from 25 to 35°C, onset gelatinization temperatures of ICI63 and ICI92 increased 2.35 and 1.81°C, respectively. These results, consistent with those reported for rice starch[9] and for wheat starch[20], indicated that starch gelatinization temperature varied with both genetic background and developmental temperature.

Sepharose CL-2B gel permeation chromatograms (Figure 2) show the molecular size distribution of starch. Distribution coefficients[44], K_o, instead of elution volumes, were used for comparison between samples. The peak retention volume of amylopectin, the first peak of the profile, was used as void volume, K_o=0, and the peak retention volume of glucose, the third peak, was used as total permeation volume, K_o=1. As developmental temperature increased from 25 to 35°C, both K_o's of amylose shifted to greater values (0.716±0.002 to 0.754±0.001 and 0.710±0.001 to 0.736±0.003 for ICI63 and ICI92, respectively), which indicated smaller amylose molecular size.

Table 5 shows the iodine affinities of starch and its components. As developmental temperature increased, iodine affinities and apparent and true amylose

contents of both starch varieties decreased. The starch iodine affinities and apparent amylose contents were in agreement with Asaoka et al.[9, 10], for rice, and Fergason and Zuber[11], for maize; however, Shi et al.[20] found that apparent amylose content of wheat increased with increased developmental temperature. The true amylose content result was consistent with that of rice starch[9, 10]. The amylose iodine affinities of ICI63 were higher than those of ICI92; however, the amylopectin iodine affinities of ICI63 were less than ICI92. The results indicated iodine affinity of starch and its components varied with variety and developmental temperature.

Figure 3 shows the chain-length distribution of debranched ICI63 and ICI92 amylopectin on Bio-Gel P6 gel permeation chromatography. The elution profiles showed three peaks and were divided into three fractions, F1-F3, in order of elution. The peak chain-lengths and the percentage of each fraction of those samples are summarized in Table 6. The first fraction of the chromatogram (F1) was a long branch-chain fraction, similar to that reported for non-waxy rice amylopectin[36, 45, 46] and for *sugary* and normal maize[47] as B3 and longer chains that stretched across three or more clusters in the amylopectin molecules[48]. The second peak, F2, was the medium branch-chain (B2) fraction that stretched across two clusters in the amylopectin molecule. The third peak, F3, of the chromatograms was the short branch-chain (B1 and A) fraction. The chain length of the F2 slightly increased as developmental temperature increased, but the chain length of F3 showed no significant difference at different temperatures. The increase in chain-length of F2 correlated with the increase of gelatinization onset

temperature (Table 4). These results agreed with those reported for rice starches[9, 10] and for taro starch[32].

Developmental temperature also affected proportions of amylopectin branch-chain fractions. The proportion of medium branch-chain, F2, increased and the proportion of short branch-chain, F3, decreased as developmental temperature increased. These results are in agreement with those for temperature effect on rice starch[9, 10]. The content of F1 correlated with the iodine affinity of amylopectin (Table 5), and this was in agreement with those reported by Takeda et al.[36] and Hizukuri, et al.[46].

Because Bio-Gel P6 can only reveal a macroview of length distribution of amylopectin branches, HPAEC-PAD was employed to show the individual components of each branch of amylopectin. Because the PAD detector response decreases when the branch-chain length increases, there was no quantitative result obtained in each individual profile. Direct comparisons between normalized detector response profiles were used to compare branch-chain length distributions, and the sum of the detector response from DP= 5 to 64 was used to normalize the profiles. The relative branch-chain length distribution of amylopectin is shown in Figure 4. ICI63 amylopectin developed at 35°C had a greater concentration between DP= 19 and 36 and a lesser concentration of DP= 7 to 16 and 41 to 49 than that developed at 25°C. According to the modified cluster model proposed by Hizukuri[48], the increased portion can be cataloged as B2 chains. Meanwhile, the branch chains of ICI92 developed at 35°C had a greater concentration between DP= 43 to 62 (equivalent to the B2 and B3 chain) and a lesser concentration of DP= 6 to 20 than that developed at 25°C. These results

indicated that the developmental temperature impact on maize amylopectin structure varied with the variety.

According to the temperature effect results on structural changes of amylopectin. there are two possible explanations to elucidate the mechanism of the effects. In a developing maize kernel, there are multiple forms of starch branching enzyme, BE I, which preferentially transfers long chains[49] with minor branching enzyme activity[50], and BE IIa and IIb, which transfer short chains with a major branching enzyme activity. High developing temperature favors BE I whose optimum temperature is 35°C[49] instead of BE IIa and IIb whose optimum temperatures are 25 and 15-20°C, respectively. Furthermore, higher developmental temperature might also weaken the double helix conformation of starch chain, which might be required for transferring branches by branching enzymes[51, 52] and decrease the branching reaction rate. These factors may cause starch developed at 35°C to have more long branches than that developed at 25°C.

In conclusion, two maize inbreds, ICI63 and ICI92, with unrelated genetic background responded differently to changing developmental temperature from 25 to 35°C. At 35°C, kernel weight and kernel density decreased, with ICI63 maintaining grain yield better than ICI92. The increased grain-developing temperature was responsible for changes in starch structure and property (increased small granules and gelatinization temperature, and decreased amylose content). ICI63 amylopectin had increased medium branch-chain fraction and decreased long and short branch-chain

fractions, whereas ICI92 had increased long and medium branch-chain fractions as developmental temperature increased.

Acknowledgments

The authors thank ICI Seeds Company, Slater, IA for the financial support.

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Table 1. Kernel Dry Weight and Kernel Density Differences by Variety and Temperature[1]

Sample		Kernel dry weight[2] (mg/kernel)	Kernel density[3] (g/ml)
ICI63	25°C	179.1±2.4[b]	1.323±0.001[a]
	35°C	155.7±2.3[c]	1.211±0.007[c]
ICI92	25°C	238.5±2.0[a]	1.299±0.001[b]
	35°C	148.0±1.7[d]	1.168±0.114[c]

[1] Figures in the same column having the same superscript are not significantly different ($P < 0.05$).

[2] Means from six subsamples of 10 kernels each for 25°C grown kernels and duplicate subsamples of 10-kernels each of four 35°C grown ears.

[3] Means from three subsamples for 25°C grown kernels and one sample each of four different 35°C grown ears.

Table 2. Proximate Composition of Maize Kernel from Different Developmental Temperatures (%)[1]

Sample	Protein[2]	Starch[2]	Moisture
ICI63 25°C	12.43±0.36[a]	69.30±1.64[a]	12.38±0.12[a]
35°C	11.82±0.06[b]	70.14±1.50[a]	12.03±0.08[a]
ICI92 25°C	12.17±0.31[ab]	64.09±1.62[b]	13.73±0.10[a]
35°C	12.57±0.21[a]	62.14±2.00[b]	13.07±0.09[a]

[1] Means and standard deviations form three subsamples; Figures in the same column having the same superscript are not significantly different ($P < 0.05$).
[2] On dry basis.

Table 3. Granular Size of Maize Starch from Different Developmental Temperatures[1]

Sample		Diameter of Equivalent Circle (μm)[2]		L/W Index[3]	
		Average	Range	Average	Range
ICI63	25°C	11.96±3.92[a]	1.27-22.68	1.19±0.15[b]	1.00-2.03
	35°C	10.45±4.47[bc]	0.20-25.34	1.24±0.23[a]	1.00-3.26
ICI92	25°C	10.78±3.18[b]	2.35-19.64	1.15±0.16[c]	1.00-2.43
	35°C	10.33±3.20[c]	1.21-18.49	1.15±0.13[c]	1.00-1.80

[1] Data form 690 starch granules; Figures in the same column having the same superscript are not significantly different ($P < 0.05$).
[2] Calculated from an area assumed to be perfect circle.
[3] Calculated by dividing maximum diameter by minimum diameter.

Table 4. Thermal Properties of Maize Starch from Different Developmental Temperatures[1]

Sample		To (°C)	Tp (°C)	Tc (°C)	Range (°C)	Enthalpy J/g	PHI
ICI63	25°C	69.11±0.29^{b}	71.97±0.28^{d}	82.10±1.40^{b}	18.59±1.24^{b}	14.28±1.02^{ab}	0.77±0.02^{a}
	35°C	71.46±0.18^{a}	74.94±0.18^{a}	87.48±2.12^{a}	20.68±1.95^{b}	15.21±0.62^{a}	0.74±0.05^{a}
ICI92	25°C	66.82±0.36^{c}	72.98±0.36^{c}	83.41±1.26^{b}	24.56±2.84^{a}	14.14±1.50^{ab}	0.58±0.03^{b}
	35°C	68.63±0.57^{b}	73.82±0.12^{b}	86.26±1.70^{a}	26.39±2.20^{a}	13.72±1.21^{b}	0.52±0.04^{c}

[1] Means and standard deviations from six measurements; Figures in the same column having the same superscript are not significantly different ($P < 0.05$);
To: Onset temperature; Tp: Peak temperature; Tc: Conclusion temperature; PHI: Peak height index.

Table 5. The Iodine Affinity of Maize Starch and Its Components from Different Developmental Temperatures[1]

Sample		IA_{starch}	$IA_{amylose}$	$IA_{amylopectin}$	Apparent Amylose (%)[2]	Amylose (%)[3]
ICI63	25°C	5.35±0.01[a]	19.29±0.15[a]	1.56±0.13[c]	28.16±0.06[a]	21.37±0.80[a4]
	35°C	4.82±0.06[d]	19.37±0.08[b]	1.41±0.01[c]	25.35±0.31[d]	18.98±0.35[b]
ICI92	25°C	5.27±0.05[b]	18.66±0.03[c]	1.82±0.04[b]	27.74±0.25[b]	20.51±0.38[a]
	35°C	5.19±0.06[c]	18.81±0.19[b]	2.14±0.02[a]	27.34±0.30[c]	18.31±0.42[b]

[1] Means and standard deviations from three measurements; Figures in the same column having the same superscript are not significantly different ($P < 0.05$); IA: iodine affinity (g/100g).

[2] Calculated from $(IA_{starch}/IA_{amylose})*100$, where $IA_{amylose}$ was assumed to be 19.0.

[3] Calculated from $[(IA_{starch}-IA_{amylopectin})/(IA_{amylose}-IA_{amylopectin})]*100$.

[4] Deviations calculated from standard deviations of starch, amylopectin, and amylose and the estimated variance was used for the Student's t-test.

Table 6. Temperature Effect on Branch-Chain of Maize Amylopectin[1]

Sample		Peak Chain Length		Weight Percentage %		
		F2	F3	F1	F2	F3
ICI63	25°C	39.9±3.5[a]	12.9±0.5[a]	6.8±1.5[a]	22.3±0.3[b]	70.8±1.8[a]
	35°C	42.1±2.9[a]	14.0±0.6[a]	5.8±1.4[a]	25.1±0.7[a]	69.1±0.6[a]
ICI92	25°C	38.7±2.9[a]	13.5±0.7[a]	7.7±1.5[a]	20.3±0.2[b]	72.0±3.3[a]
	35°C	41.9±2.6[a]	13.1±0.7[a]	8.1±2.2[a]	21.8±1.5[b]	70.1±2.7[a]

[1] Means and standard deviations from three or four measurements; Figures in the same column having the same superscript are not significantly different ($P < 0.05$); The fractions, F1 to F3, are corresponding to Figure 4.

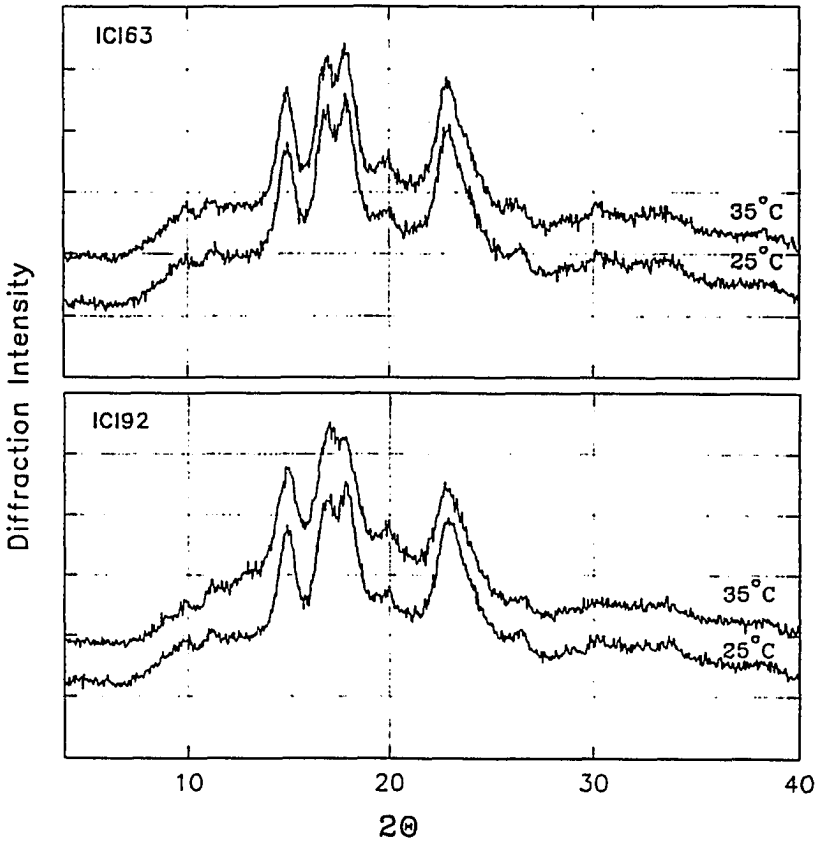

Figure 1. X-Ray diffractogram of maize starch from ears developed at different temperatures. The signals recorded from diffraction angle (2θ) 4 to 40 degrees.

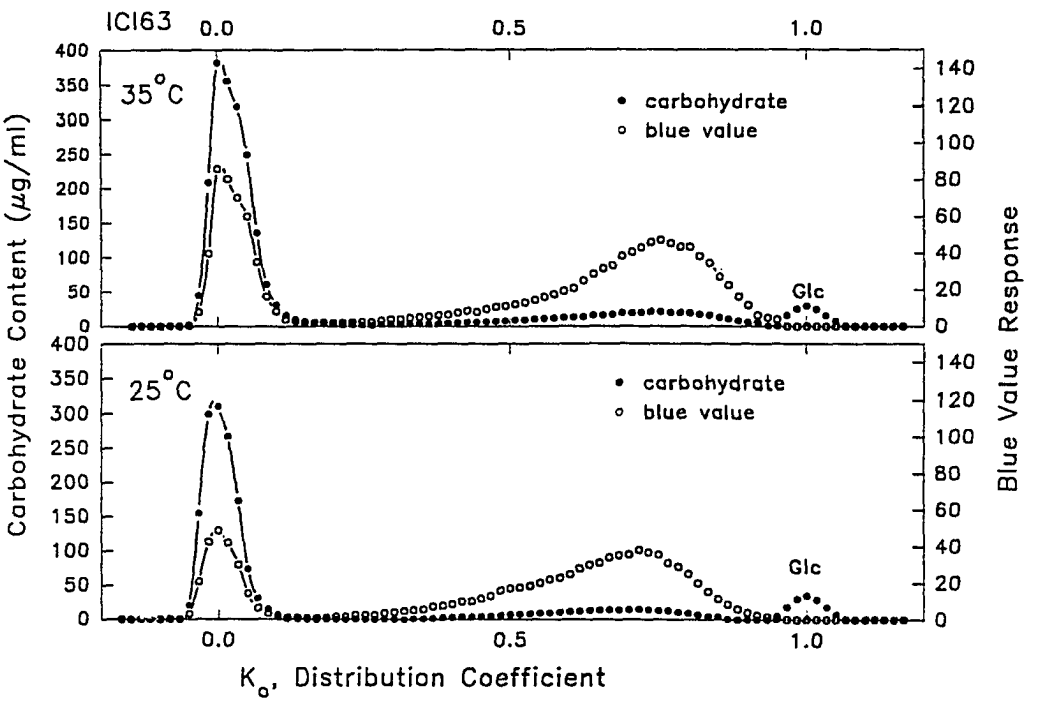

Figure 2a. Sepharose CL-2B chromatograms of ICI63 maize starches developed at different temperatures. Glucose (Glc) was used as the marker.

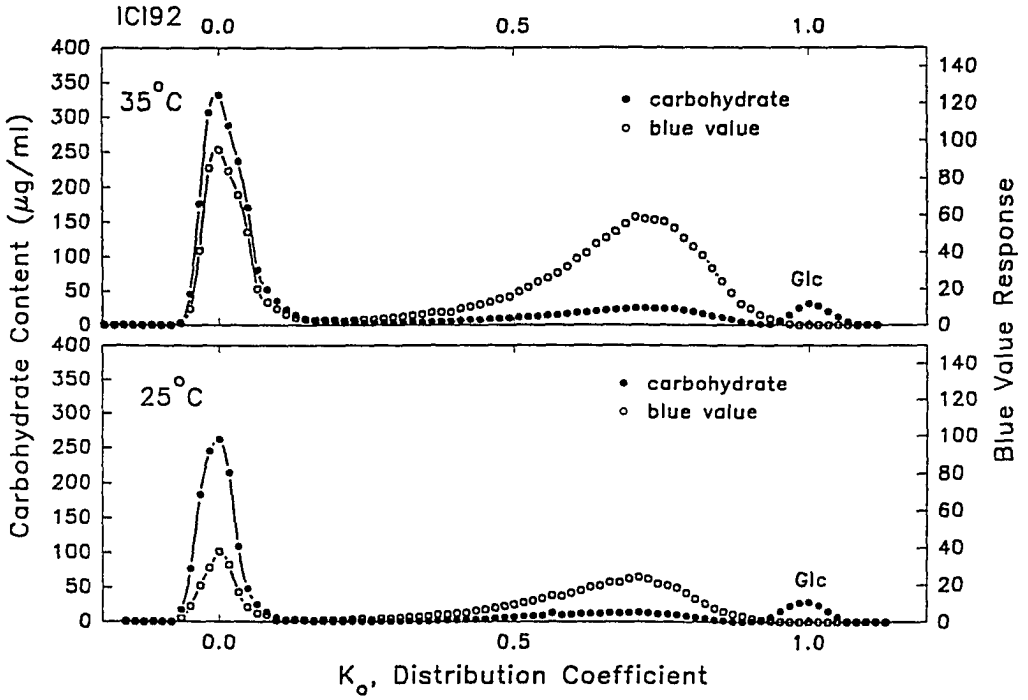

Figure 2b. Sepharose CL-2B chromatograms of ICI92 maize starches developed at different temperatures. Glucose (Glc) was used as the marker.

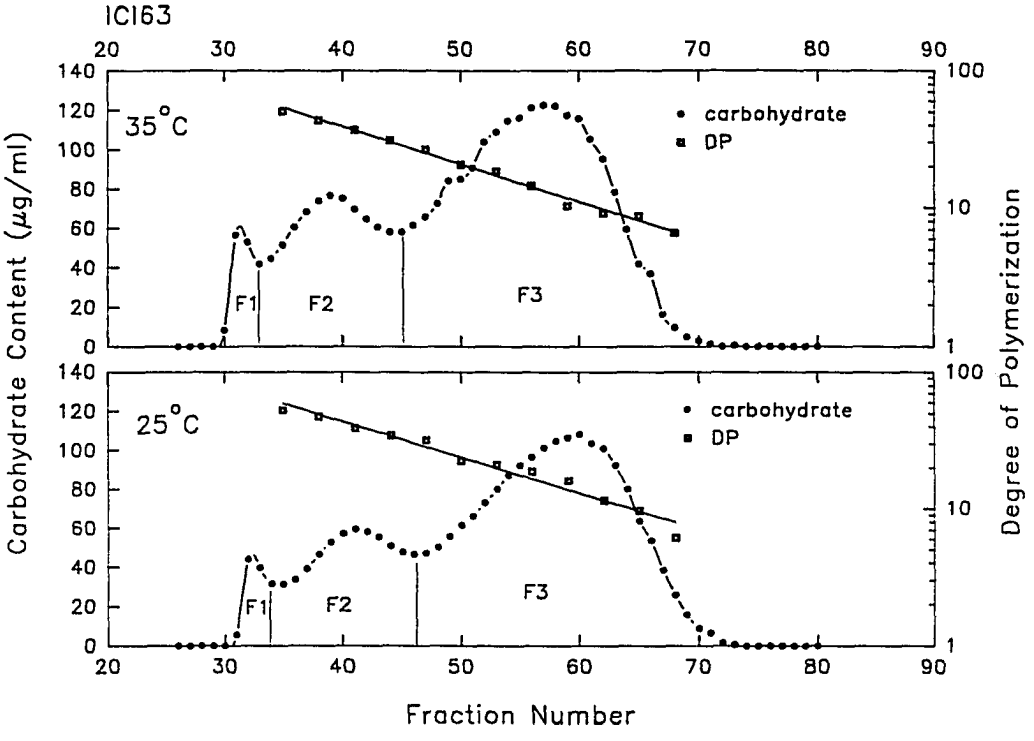

Figure 3a. Bio-Gel P6 chromatogram of isoamylase debranched ICI63 maize amylopectin (DP= degree of polymerization).

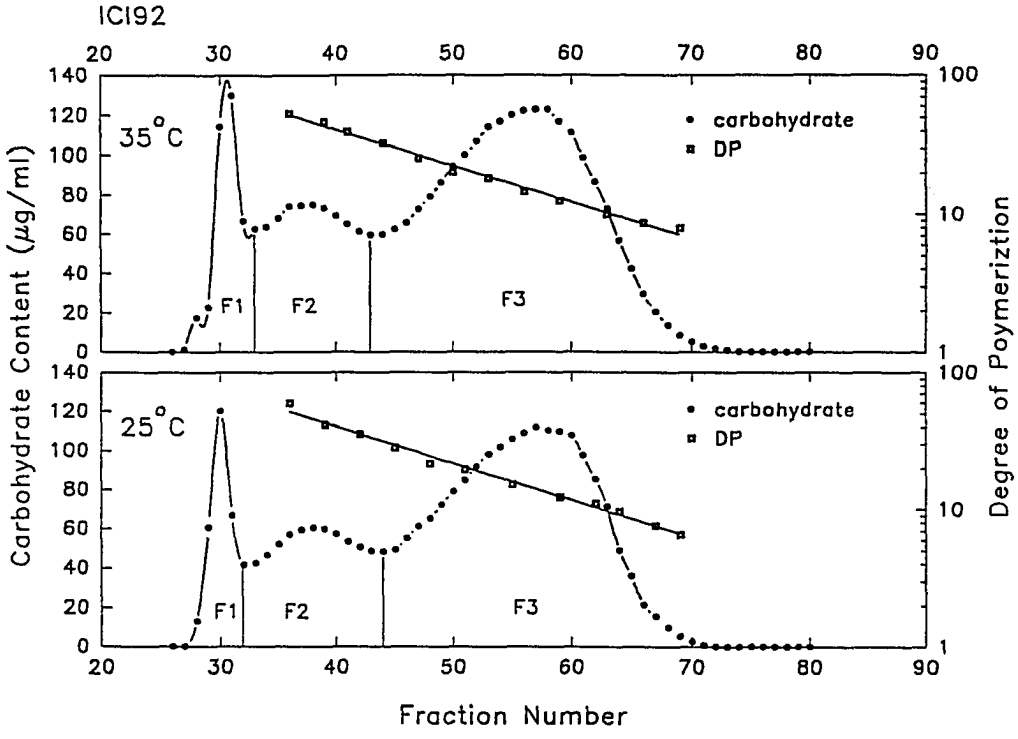

Figure 3b. Bio-Gel P6 chromatogram of isoamylase debranched ICI92 maize amylopectin (DP= degree of polymerization).

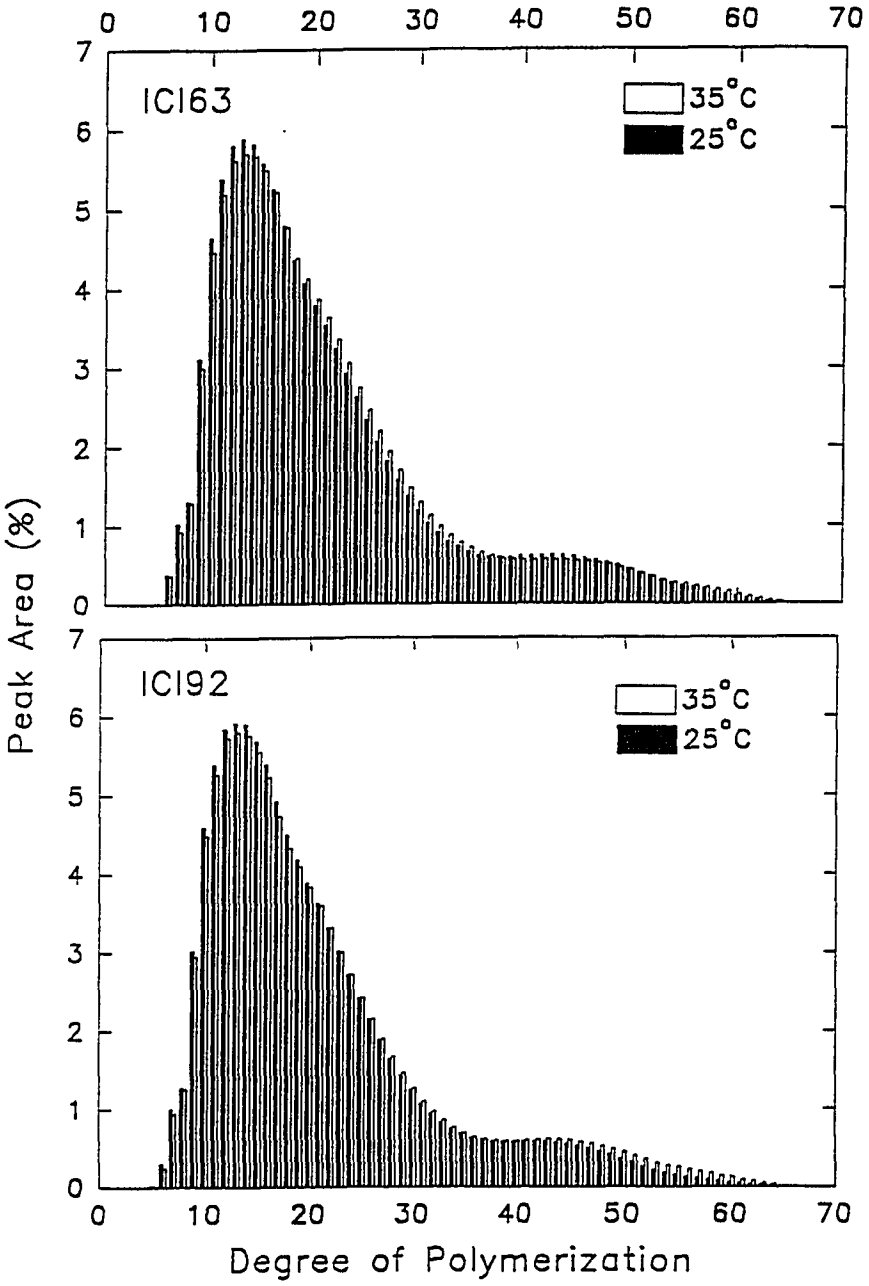

Figure 4. Temperature effect on branch-chain length distribution of ICI maize amylopectin analyzed by HPAEC-PAD (mean of three measurements).

TEMPERATURE EFFECT ON RETROGRADATION OF AMYLOSE SOLUTION[1]

A paper to be submitted to Carbohydrate Research

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Abstract

Commercial potato amylose was used to examine the temperature effects on retrogradation of diluted amylose solutions. Amylose solutions were prepared in two different ways, water dispersion and potassium hydroxide solubilization. The retrogradation rate decreased as incubation temperature increased (5 to 45°C). For a 3.5 mg/mL water-dispersed amylose solution, initial retrogradation rate decreased from 58.8 to 7.1% as incubation temperature increased. After the solution was incubated at 25°C for 100 days, ~ 50% of amylose molecules with small molecular weight (DP_n 180 and DP_w 290) precipitated out from solution. The chain length of retrograded amylose

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[1] Journal Paper No. J-#### of the Iowa Agriculture Experiment Station, Ames, Iowa. Project No. 3258.

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crystallites increased from DP_n 39 to 52 and DP_w 47 to 72 as incubation temperature increased from 5 to 45°C. Three different concentrations of amylose solutions (10, 7 and 4 mg/mL) were prepared by using potassium hydroxide and then neutralized with hydrochloric acid. Initial retrogradation rate decreased (84.9 to 7.4 %) as amylose concentration decreased and as incubation temperature increased in the 0.1 M potassium chloride solution. The potassium hydroxide solubilization procedure and the presence of potassium chloride retarded the retrogradation rate and affected the chain length of retrograded amylose crystallite.

Introduction

Starch retrogradation has an important effect on the texture of many starch-containing foods, *e.g.*, the staling of bakery products and the eating quality of cooked rice. The retrogradation process has been used for fractionating amylose from starch[1, 2], and preparing bulking agents (dietary fiber), known as resistant starch[3]. The process is also required for branch formation during starch biosynthesis[4-6].

Retrogradation is a process in which gelatinized starch molecules reassociate to form an ordered structure (double helix)[7-10]. In its initial phases, two or more starch chains may form a simple juncture point which then may develop into more extensively ordered regions[10-12]. The retrogradation process can happen on both amylose and amylopectin fractions[9, 10, 12]. Amylopectin retrogradation is reversible[13-15], but amylose retrogradation is essentially irreversible at temperatures below 100°C[10, 16-18]. Therefore,

it is extremely difficult to redissolve retrograded amylose without dimethyl sulfoxide or alkali.

Amylose is essentially a linear molecule with few branches. Like other linear polymers, amylose molecules have a strong tendency to crystallize. In aqueous solution, amylose molecules rapidly associate to build up molecular aggregates that soon exceed colloidal dimensions and precipitate[1, 12, 19]. The aggregation process is rapid, and the molecular size of amylose is too large to produce perfect crystallites[20, 21]. As a result, the precipitate is a mixture of crystalline and amorphous regions as indicated by X-ray diffraction patterns and acidic or enzymatic hydrolysis[7, 12, 22]. Retrograded amylose precipitate shows an A- or B-type X-ray pattern that depends on the retrogradation conditions[22-29]. The crystalline region can be 30 to 65% of the total amylose gel, and the amount differs with the retrogradation conditions[22, 30]. This crystalline region is resistant to acidic and enzymatic hydrolysis[7, 22, 30-32]. The conformation of the resistant crystalline region is proposed to be double helices interspersed with amorphous regions[24, 30, 33] similar to the starch chains in starch granules[7, 8, 34, 35]. The chain length of the crystalline region from different studies varies[22, 30, 36].

The retrogradation rate of amylose is dependent on molecular size, temperature, concentration, pH, and the presence of other chemical agents in the solution[37-39]. It has been shown that amylose from different starch sources retrograde at different rates, depending upon their average molecular weight[36, 37, 39-41]. Amylose with DP 80 to 100 has the highest retrogradation tendency[42-45]. Temperature is negatively correlated with retrogradation rate[37]. Suzuki et al.[46] reported that an increase of incubation temperature

temperature (0 to 30°C) of a soluble starch solution resulted in an increase of the thermal transition temperature of retrograded starch. High pH deprotonates the hydroxyl group of amylose, and charge repelling inhibits the retrogradation process. Sugars, as additives, increase retrogradation rate, whereas salts affect the rate of retrogradation differently, depending on the salt species and concentration[37, 47-53]. Surfactants, lipids and long chain alcohols can form complexes with amylose[54-60] and prevent or inhibit retrogradation.

The double helical conformation similar to the retrograded amylose is proposed as a requisite for branch formation during starch biosynthesis[4-6]. The glucan transfer reaction catalyzed by branching enzyme is temperature dependent[61, 62]. Temperature affects not only branching enzyme activities but also substrate conformation. At higher temperature, double helix formation rate is slower and there are more possibilities to form longer crystalline fragments. In this study, dilute amylose solutions were used as a model system to investigate the temperature effects on double helical conformation of starch chains. In addition to the branching enzyme activity alternation[63], this study aimed to provide an explanation for amylopectin developed at high temperature contained a higher proportion of long branch-chain[64-66].

Experimental

Materials. Potato amylose (Type III) and porcine pancreatic α-amylase were purchased from Sigma Chemical Co. (St. Louis, MO). Pullulan molecular weight standards (Shodex Standard P-82 kit, Showa Denko K.K., Tokyo, Japan) were purchased from Millipore Waters (Mildord, MA). The molecular weights and polydispersities of the standards were: 853,000, 1.14; 380,000, 1.12; 186,000, 1.13; 100,000, 1.10; 48,000, 1.09; 23,700, 1.07; 12,200, 1.06; 5,800, 1.07. Maltotetradecaose was purchased form Nakano Vinegar Co. (Aichi, Japan) and maltoheptaose and maltose was from Aldrich Chemical Co. (Milwaukee, WI). Mlatotetradecaose, maltoheptaose and maltose were used as oligosaccharide standards. Other chemicals were reagent grade, purchased from Fisher Scientific (Springfield, NJ) and used without further treatments.

Preparation of retrograded amylose. Two methods were used to prepare amylose solutions.

A. Water dispersion of amylose: Potato amylose was wetted with a few drops of methanol before being dispersed in water. The aqueous suspension was maintained at 85-90°C with continuous stirring for 6 hr to dissolve amylose and to evaporate the methanol (some residual butanol present in commercial amylose preparation might be removed). To remove insoluble substances, the solution was filtered through Whatman No. 4 filter paper. The amylose solution was dispensed into glass vials and autoclaved

at 125°C for 30 min for sterilizing and then incubated at 5, 15, 25, 35 and 45°C for periods of time to facilitate retrogradation of amylose.

B. Potassium hydroxide solubilization of amylose: Potato amylose was dispersed in 0.5 N potassium hydroxide and agitated with a stirring rod to avoid the formation of insoluble lumps. The mixture was placed in a refrigerator (4°C) with occasional stirring until it became a clear solution. The solution was then neutralized by using 0.5 N hydrochloric acid and diluted to designated concentrations (4, 7, and 10 mg/mL). The final concentration of potassium chloride was at 0.1 M and at pH 6.0. Amylose solutions were dispensed into glass vials and autoclaved at 125°C for 30 min for sterilizing and then incubated at 5, 15, 25, 35, and 45°C for extended time periods to facilitate formation of retrograded amylose precipitate.

Preparation of amylose crystallite. Amylose crystallite was obtained by hydrolyzing retrograded amylose with porcine pancreatic α-amylase or with 16% sulfuric acid at 25°C for 30 days (manually shaken everyday)[30]. The crystallites remaining after enzyme and acid treatments were washed with deionized water and collected by centrifuging at 6,700 xg, for 15 min (JA-21 Beckman Instruments, Fullerton, CA). The crystallite was solubilized in dimethyl sulfoxide (DMSO) (90%) and heated in boiling water for 20 min. The chain length of the crystallite was determined by using high-performance liquid chromatography-size-exclusion chromatography (HPLC-SEC).

Molecular weight of potato amylose. Molecular weight of potato amylose was determined by measuring its intrinsic viscosity and by using HPLC-SEC.

A. Intrinsic viscosity method: Viscosity average degree of polymerization (DP_v) of the amylose was determined by measuring intrinsic viscosity. Intrinsic viscosity of potato amylose was determined by using a Cannon-Fenske capillary viscometer (Fisher Scientific, Springfield, NJ) in both 0.5 M potassium hydroxide and dimethyl sulfoxide. The empirical Mark-Houwink constants, K and α, were 8.50×10^{-3}, 0.76 for potassium hydroxide and 3.06×10^{-2}, 0.64 for dimethyl sulfoxide, respectively, and were used for calculating the molecular weight of amylose[67].

B. HPLC-SEC method: Potato amylose was dispersed in 90% dimethyl sulfoxide and heated in a boiling water bath for 20 min and then stirred at room temperature overnight and filtered through a 0.45 μm nylon membrane before being injected into the chromatographic system. An HP 1050 Series Pump System equipped with a 20 μL sample loop and an HP 1047A Refractive Index Detector (Hewlett Packard, Wilmington, DE) was used for the analysis. An Ultra-Ware Integrated HPLC Mobile Phase Handling System (Vineland, NJ) was employed to filter, sparge, and pressurize the eluent with helium. The mobile phase used was deionized water from a Milli-Q water system (Millipore Co., Bedford, MA) and filtered through a 0.2 μm nylon membrane. Potato amylose was analyzed by three sequentially connected columns of TSK-GEL (G6000PWXL, G4000PWXL and G3000PWXL (300 x 7.8 mm)) with a PWXL Guardcolumn (40 x 6 mm) (Tosohaas, Montgomeryville, PA) at 80°C and a 0.6 mL/min flow rate. The refractive index detector was maintained at 50°C.

Chromatographic data were collected and processed on a NEC computer with Maxima 820 gel permeation chromatographic software (Millipore, Waters Chromatography Div., Milford, MA). The narrow standard calibration was performed by using pullulan and malto-oligosaccharide standards[68, 69]. A cubic standard curve was calculated using log molecular weight versus standard retention time. For the determination of the molecular weight averages, the chromatogram was divided into a number of slices and the molecular weight of slices was determined by calibration curve. The slice interval was set at 10 sec and the molecular weight distribution was assumed to be monodisperse[69]. The number- and weight-average molecular weight (M_n and M_w) were calculated by following their definition[69-76]:

$$M_w = \frac{\sum (A_i \times M_i)}{\sum A_i} \qquad M_n = \frac{\sum A_i}{\sum (A_i / M_i)}$$

In the equations, A_i is the area of slice i and M_i is the molecular weight of slice i. Degree of polymerization (DP) was calculated as molecular weight divided by 162. Polydispersity was used to describe the molecular weight distribution of polysaccharide and was defined as[69]:

$$D = \frac{M_w}{M_n}$$

Rate of amylose retrogradation. The rate of amylose retrogradation was determined by measuring the decrease in amylose concentration in solution as retrogradation proceeded. The amylose concentration was determined by measuring the total carbohydrate content of the supernatant using the phenol-sulfuric acid method[77]. The retrograded amylose was removed from the supernatant solution by centrifuging at 11,500 xg for 10 min (Model 59A Microcentrifuge, Fisher Scientific, Springfield, NJ).

Morphology of the retrograded amylose. The microstructure of retrograded amylose prepared by the water dispersion method was observed by using JEOL JSM-35 scanning electron microscope (JEOL Ltd., Tokyo, Japan). The lyophilized retrograded amylose gel was attached to a specimen stud, coated with gold-palladium alloy, and representative micrographs were taken at a 10,000x magnification.

Molecular weight of amylose crystalline fragment. Chain length of retrograded amylose crystalline fragment was determined by using HPLC-SEC with two sequentially connected columns of TSK-GEL (G4000PWXL and G3000PWXL) with the PWXL Guardcolumn as previously described at a 50°C with 0.5 mL/min flow rate. Other operating conditions were the same as previously described.

Results and Discussion

The molecular weight and distribution profile of potato amylose determined by using capillary viscometry and HPLC-size exclusion chromatography (SEC) are shown in Table 1 and Figure 1. The amylose intrinsic viscosity was smaller than that reported by Greenwood[21] but similar to that reported by Foster[78]. Potato amylose degree of polymerization determined by using viscometry (DP_v) were 1,900 and 2,060 in 0.5 N potassium hydroxide and dimethyl sulfoxide, respectively. The number- and weight-average of degree of polymerization (DP_n and DP_w) obtained by HPLC-SEC was 250 and 1490, respectively. The DP values might be underestimate by using HPLC-SEC because amylose had a slightly branched structure and a random coil conformation which resulted in a less extended molecule compare to pullulan standards[79, 80]. It was reported that the molecular weights of amylose specimens are about 35% larger than those of the pullulan specimens eluted at the same elution volume by HPLC-SEC[80]. The polydispersity of the potato amylose molecule was 5.953, which was similar to a typical synthetic polymer value[72]. To prevent entanglement between amylose molecules, the amylose was dissolved in 90% dimethyl sulfoxide before being injected to the chromatographic system. The dimethyl sulfoxide was separated from amylose and was eluted at the end of the profile.

To avoid effects of other chemicals, *e.g.* potassium chloride, a diluted water-dispersed amylose solution (without using alkaline solution) was prepared and used for the study. The original solution concentration determined by using phenol-sulfuric acid

method was 3.5 mg/mL. The retrogradation time course was shown in Figure 2. The incubation temperature significantly affected the retrogradation rate of amylose solution, which agreed with that reported by Whistler[37]. In the solution incubated at 5°C, most of amylose molecules rapidly retrograded and precipitated from solution within the first 9 days. At the end of the 9th day, 78% of amylose precipitated out. After 9 days, amylose continued to retrograde at a slower rate. The solution incubated at 15°C followed a similar pattern, but the retrogradation rate was slower than that incubated at 5°C. On the 85th day, retrogradation at 15°C only reached 71% and was expected to continuously increase. For the solution incubated at 25°C, the retrogradation rate was much slower than those at 5 and 15°C, and the retrogradation pattern was different. About 12.5% of amylose retrograded within the first day and followed with a 17-day lag period where little additional retrogradation occurred. After the lag period, amylose continued to retrograde at a faster rate and reached 45% on the 85th day, and retrogradation was expected to continue. The lag period is attributed to the time required for nucleation process[81]. According to the retrogradation time course pattern, there were two main stages in amylose retrogradation with different rates, which the rates of the first stages were faster than the one of the second stage. The retrogradation percentage of first stage was decreased as incubation temperature increased. The percentages of retrogradation for the first stage were 78, 56, 18% for 5, 15, 25°C, respectively.

For the solutions incubated at higher temperature, less than 10% of amylose retrograde within the first day, *i. e.* 9.2 and 7.1% for 35 and 45°C, respectively, and

after that no increase in retrogradation was found up to the 85th day. The correlations between the incubation time and the percentage retrogradation at the high temperatures were low (correlation coefficient, r^2= 0.28 and 0.04 for 35 and 45°C, respectively). The retrogradation rate within the first 24 hr was defined as initial retrogradation rate and are shown in Table 2. The initial retrogradation rate well reflected the decrease of retrogradation as the temperature increased.

To study the molecular profile of remaining amylose, the supernatant after 100-day incubation was subjected to HPLC-SEC analysis. The molecular profiles of remaining amylose in the supernatant at different temperatures are shown in Figure 3. At 5°C, most amylose molecules were precipitated only with a small amount of large molecules remaining in the solution. Those molecules which remained could be amylopectin impurity or entangled amylose. At 15°C, most amylose molecules also retrograded and precipitated out. There were a substantial amount of large molecules eluted at the void volume that remained in the supernatant. The amount of molecules eluted at the void volume was greater than that in the original solution. The increase in large molecules was attributed to entangled amylose molecules. At 25°C, about half the amylose retrograded and the other half remained in the supernatant. It was plausible that during amylose retrogradation, the molecules initially entangled together and the aggregates[42] eventually precipitated out. At 35 and 45°C, most of the molecules remained in solution, but the entangling phenomenon was obvious. The last peak of the chromatogram was attributed to methanol residue which was used for dispersing amylose or butanol residue that was present in the amylose sample.

By comparing the molecular profiles of supernatant incubated at different temperatures (Figure 3), retrogradation was a selective process based on molecular size. The fraction of small molecules retrograded preferably and precipitated first. To determine the molecular weight of the fraction which had a high tendency to retrograde, the chromatogram of the original amylose was subtracted by that of the supernatant after 100-day incubation. The difference between the chromatograms of the original and the supernatant after 100 days at 25°C is shown in Figure 4. The differential chromatogram showed that the portion above the baseline was the molecules that aggregated and became precipitation or entangled molecules, and the portion below the baseline represents those developed through entanglement, during the incubation. The DP_n, DP_w and polydispersity of this portion were 180, 290 and 1.6213, respectively. The results showed that small size amylose molecules had a higher tendency to retrograde. This is consistent with amylose of DP 80-100 having a greater tendency to retrograde[43-45].

The morphology of retrograded amylose differed with incubation temperature. At lower temperature (5, 15, 25°C), amylose formed a gel and precipitated out from the solution (Figure 5). The network structure of amylose gel was better constructed as the incubation temperature increased, because the gel formation at higher temperature required more time and this allowed the amylose molecules to align over a longer period. In the amylose solutions incubated at 35 and 45°C, a small amount of fiber-like precipitate formed instead of gel. It is proposed by Greenwood[21] and Gidley and Bulpin[45] that chain lengths of amyloses have a profound effect on the aggregation of amylose in aqueous solution. Amyloses with small chain lengths (less than 110) have

predominant behavior of precipitation over gelation which tends to occur particularly for higher chain lengths. For short chain amylose, chain alignment was dominate and eventually led to precipitation instead of gelation[21, 45].

The chain length distribution of the retrograded amylose crystallite was examined by the HPLC-SEC analysis. A size-exclusion chromatogram of amylose crystallite from retrograded amylose at 5°C was shown in Figure 6. The chain length distribution of the crystallite showed a narrow polydispersity, and the values varied between 1.215 and 1.381. The polydispersity of amylose crystallite showed an increased trend as the incubation temperature increased. The temperature effect on crystalline chain length was obvious. The DP_n increased from 39 to 52 and DP_w increased from 47 to 72 when temperature increased from 5 to 45°C (Table 3). The data may provide a structure to illustrate the result of Suzuki et al.[46] that the thermal transition temperature of retrograded soluble-starch is higher for the starch retrograded at higher temperature. The crystallite chain length prepared at 5°C was in agreement with that reported by Jane and Robyt[30]. The values of crystallite chain length prepared at 5°C determined by HPLC-SEC were in good agreement with the one determined by modified Park-Johnson method[79, 82]. The results indicated the difference in hydrodynamic volume of crystallite and pullulan is negligible because of the small molecular size and lack of branch points on the crystallite produced in this study.

To prepare more concentrated amylose solutions, amylose was dispersed in a potassium hydroxide solution followed by neutralization with hydrochloric acid solution. Retrogradation rates of amylose solutions prepared by the potassium hydroxide

solubilization procedure are shown in Figure 7. Temperature[37] and amylose concentration[37, 38, 41] are known to significantly affect the retrogradation of amylose solution. Retrogradation rate increased as the amylose concentration increased and the incubation temperature decreased. When an amylose solution (10.8 mg/mL) incubated at 5°C, amylose molecules rapidly retrograded and precipitated from the solution within the first 3 days. By the 3rd day, 93% of the amylose precipitated out. After the 3rd day, amylose continued to retrograde at a slower rate. The retrogradation reached a plateau after 25 days, and most of the amylose (99.5%) was precipitated. The solution incubated at 15°C followed a similar pattern, except the retrogradation rate was slower than that at 5°C, and more time was needed to reach the plateau. The solution incubated at 25°C retrograded much slower than 5 and 15°C, and the reaction pattern was slightly different. After 33 days, the retrogradation rate decreased but did not reach a plateau up to day 101. The retrogradation pattern of the solution at 35°C was different from those at 5, 15 and 25°C, with 14.9% of the amylose retrograded within the first day. After day one, a 24-day lag period followed and the retrogradation of amylose did not significantly increase. After the lag period, amylose continued to retrograde but did not follow a linear pattern. The reason for stepwise retrogradation pattern is not clear. One possible explanation was that different fractions of molecules with different retrogradation tendency retrograded in different incubation periods. Another possibility was that retrograded amylose double helixes were aggregated into larger and denser gel and precipitated by batch. For the solutions incubated at 45°C, there was no correlation (correlation coefficient, $r^2 = 0.05$) between the incubation time

and the percentage of retrogradation. Within the first day, 11.9 % of the amylose retrograded, and after that no increase in retrogradation was found up to 101 days. The retrogradation of the solution was inhibited at 45°C; however, 11.9% of the amylose molecule had a very high tendency to retrograde.

The retrogradation rate decreased as amylose concentration decreased. The retrogradation of the 7.0 mg/mL solution at 5°C reached the plateau after 55 days. The retrogradation of the solution at temperature higher than 15°C did not reach a plateau after 103 days. The retrogradation rate of the 4.1 mg/mL amylose solution was significantly lower than those of more concentrated solutions. The amylose solution at 5°C rapidly retrograded and precipitated from solution after 11 days. On the 11th day, 70.7% of the amylose molecules was found precipitated from aqueous solution. After the 11th day, amylose continued to retrograde at a slower rate. The solutions incubated at 35 and 45°C did not show a correlation ($r^2 < 0.1$) between incubation time and the percentage retrogradation. A fraction of about 10% with a high tendency to retrograde within first day was found in all the solutions, regardless of the sample concentration and incubation temperature.

Initial retrogradation rate was determined to investigate the retrogradation of amylose solutions. The initial retrogradation rate correlated well with sample concentration and incubation temperature (Table 2). The initial retrogradation rate decreased as amylose concentration decreased and incubation temperature increased. At 5°C incubation temperature, the concentration effect was more pronounced than that at a higher temperature. The potassium hydroxide solubilization procedure changes the

conformation of amylose through charge repelling and generated potassium chloride as a result of neutralization. The conformation change could retard the retrogradation rate of amylose, comparing with those amylose solutions at similar concentration (3.5 and 4.1 mg/mL) (Table 2, Figure 2 and 7c). The initial retrogradation rate of the solution without alkaline dispersion decreased from 58.8 to 18.1% (Table 2). This result is in agreement with the reports of Kitamura et al.[25] and Suzuki et al.[39].

The crystalline chain length profile showed a narrow distribution (Figure 8). The DP_n ranged from 27 to 50 and DP_w from 37 to 57, and varied with incubation temperature and amylose concentration (Table 4). The chain length from 10.8 and 7.0 mg/mL solution were about 32 and 45 for number- and weight-average DP, respectively. There were two exceptions, for 7.0 mg/mL at 35°C and 10 mg/mL at 45°C, in which chain length was about 27 and 37 or 38 for DP_n and DP_w, respectively. The DP of crystallites from diluted amylose solution (4.1 mg/mL) showed trend toward higher values as the incubation temperature increased (Table 4). The DP_n increased from 34 to 50 and DP_w increased from 45 to 57 as the temperature increased from room temperature or below (25°C) to 45°C.

The retrogradation rates of diluted amylose solutions decreased as concentrations decreased and incubation temperature increased. The alkaline-dispersion procedure and the presence of potassium chloride retarded the retrogradation rate. There was an interaction effect of the concentration and the incubation temperature on retrogradation rate. In 3.5 mg/mL water-dispersed amylose solutions incubated at 25°C for 100 days, small molecular weight with DP_n 180 and DP_w 290 precipitated out from the solution.

The chain length of retrograded amylose crystallites from water-dispersed amylose solution increased from DP_n 39 to 52 and from DP_w 47 to 72, as incubation temperature increased from 5 to 45°C. The effect of the potassium hydroxide-dispersion procedure and the presence of potassium chloride on the chain length of retrograded amylose crystallites did not provide a linear trend.

Acknowledgment

The authors thank ICI Seeds Company, Slater, IA, for the financial support and the valuable advice from Dr. Peter L. Keeling.

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Table 1. Molecular Size of Potato Amylose Determined by Various Methods[1]

Methods	[η]	DP_v	DP_n	DP_w	DP_{peak}	Poly-dispersity[2]
$Viscometry_{KOH}$	125	1900	---	---	---	---
$Viscometry_{DMSO}$	105	2060	---	---	---	---
HPLC-SEC[3]	---	---	250	1490	430	5.953

[1] [η]: intrinsic viscosity (ml/g) measured in 0.5 N potassium hydroxide and dimethyl sulfoxide ; DP: degree of polymerization; DP_v calculated from intrinsic viscosity; DP_n, DP_w, DP_z and DP_{peak} stand for number-, weight-, z-average and peak DP.
[2] Polydispersity: DP_w/DP_n
[3] Pullulan standards were used for molecular weight calibration.

Table 2. Initial Retrogradation Rate (%) of Amylose Solution in First Day[1]

Temp. mg/mL	5°C	15°C	25°C	35°C	45°C
Water dispersed amylose[2]					
3.5[2]	58.8±3.3	17.4±0.9	12.5±0.8	9.2±1.2	7.1±0.4
Potassium hydroxide solubilized amylose[3]					
4.1[3]	18.1±1.0	10.1±0.7	10.3±0.7	9.1±0.3	7.4±0.6
7.0[3]	58.9±1.3	31.1±3.9	13.5±0.9	12.4±0.3	11.1±0.5
10.8[3]	84.9±0.1	47.0±2.6	17.6±1.1	14.9±0.6	11.9±1.3

[1] Mean and standard deviation of duplicate samples.
[2] Amylose was dispersed in water and heat in boiling water bath.
[3] Amylose was dispersed in potassium hydroxide and neutralized with hydrochloric acid; the final solution contained 0.1 M potassium chloride.

Table 3. Chain Length of Retrograded Amylose Crystallite Prepared by Water Dispersion Procedure at Different Incubation Temperature[1]

Temp. °C	Total retrogradation %[2, 3]	DP_n	DP_w	DP_{peak}	Polydispersity
5	88.3±4.5	39±2	47±2	43±2	1.215±0.006
15	70.8±0.9	40±1	50±2	45±2	1.241±0.012
25	44.8±3.2	44±2	54±2	47±1	1.232±0.031
35	14.2±2.6	45±2	58±3	51±3	1.294±0.012
45	8.6±0.4	52±3	72±6	52±1	1.381±0.058

[1] Means and standard deviations of duplicate samples for total retrogradation and triplicate samples for chain length determination.
[2] Original amylose solution concentration was 3.5 mg/ml.
[3] Retrograded amylose semicrystalline was obtain by using α-amylase hydrolysis.
[4] After 85 days.

Table 4. Chain Length of Retrograded Amylose Crystallite Prepared by Potassium Hydroxide/Hydrochloric Acid Procedure at Different Incubation Temperature[1]

Conc. mg/ml	Temp. °C	Total retrogradation %[2, 3]	DP_n	DP_w	DP_{peak}	Poly-dispersity
4.1	5	96.6±2.0	34±1	45±2	44±1	1.350±0.010
4.1	15	61.0±4.5	33±1	44±2	42±2	1.354±0.016
4.1	25	11.6±0.5	33±3	44±3	42±4	1.353±0.051
4.1	35	10.3±0.1	43±0	51±1	56±1	1.193±0.019
4.1	45	8.2±0.8	50±3	57±3	59±4	1.125±0.027
7.0	5	98.6±0.2	32±3	44±3	42±3	1.363±0.018
7.0	15	85.4±1.7	33±1	46±1	43±1	1.404±0.018
7.0	25	55.0±0.2	31±2	45±3	39±2	1.419±0.015
7.0	35	48.0±2.2	27±2	38±3	31±2	1.412±0.032
7.0	45	20.4±0.1	32±3	45±4	42±3	1.424±0.039
10.8	5	99.7±0.1	31±3	45±4	41±4	1.430±0.012
10.8	15	89.8±0.1	32±2	45±4	42±4	1.433±0.020
10.8	25	75.7±0.1	32±2	47±4	41±3	1.454±0.022
10.8	35	43.3±0.6	32±2	47±3	39±3	1.456±0.029
10.8	45	11.1±1.1	27±2	37±3	31±3	1.397±0.027

[1] Means and standard deviations of duplicate samples for total retrogradation and triplicate samples for chain length determination.

[2] Retrograded amylose crystallite was obtain by using 16% sulfuric acid hydrolysis for 30 days.

[3] After 104 days for concentrations of 4.1 and 7.0 mg/mL; and 101 days for concentration of 10.8 mg/mL.

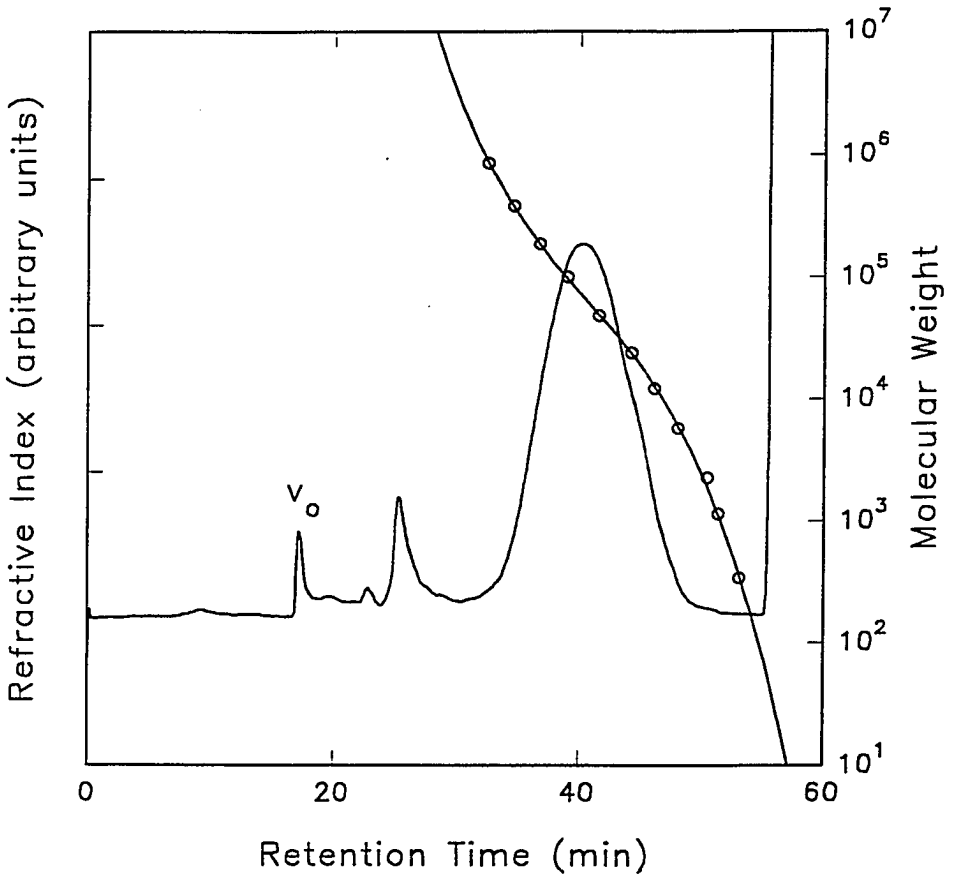


Figure 1. Chromatogram of potato amylose on the HPLC-SEC. The -o- was molecular weight calibration curve of pullulan standards. V_o indicated the void volume of the column.

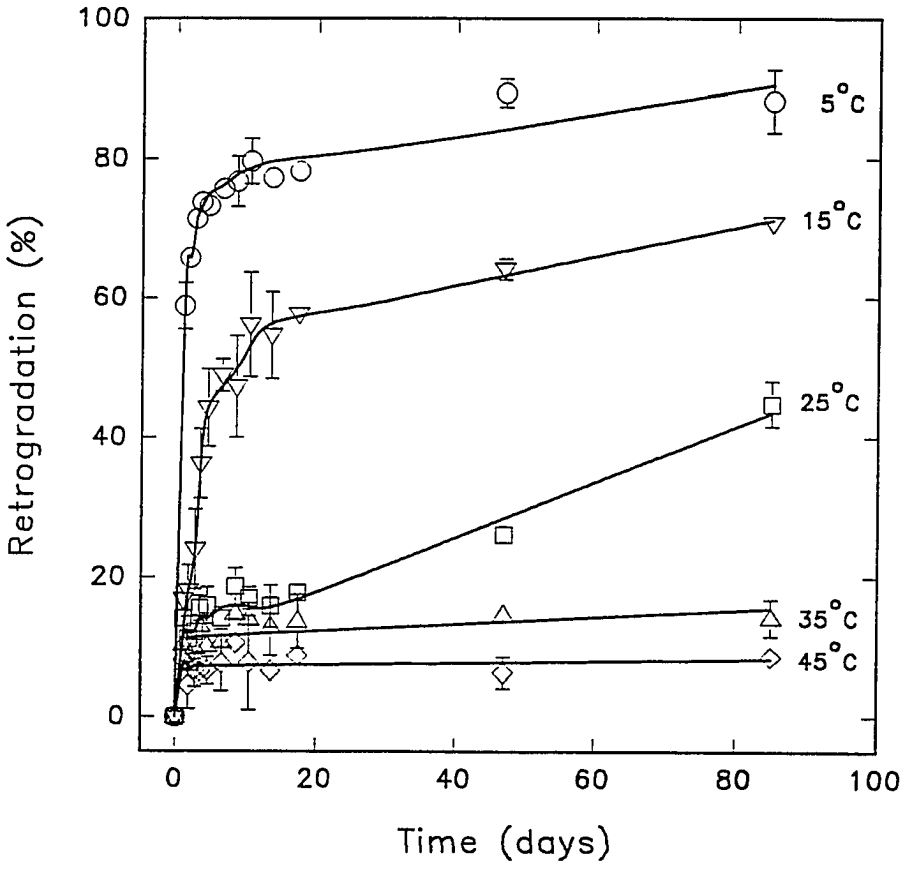


Figure 2. The time course of amylose solution retrogradation. The original amylose concentration was 3.5 mg/mL.

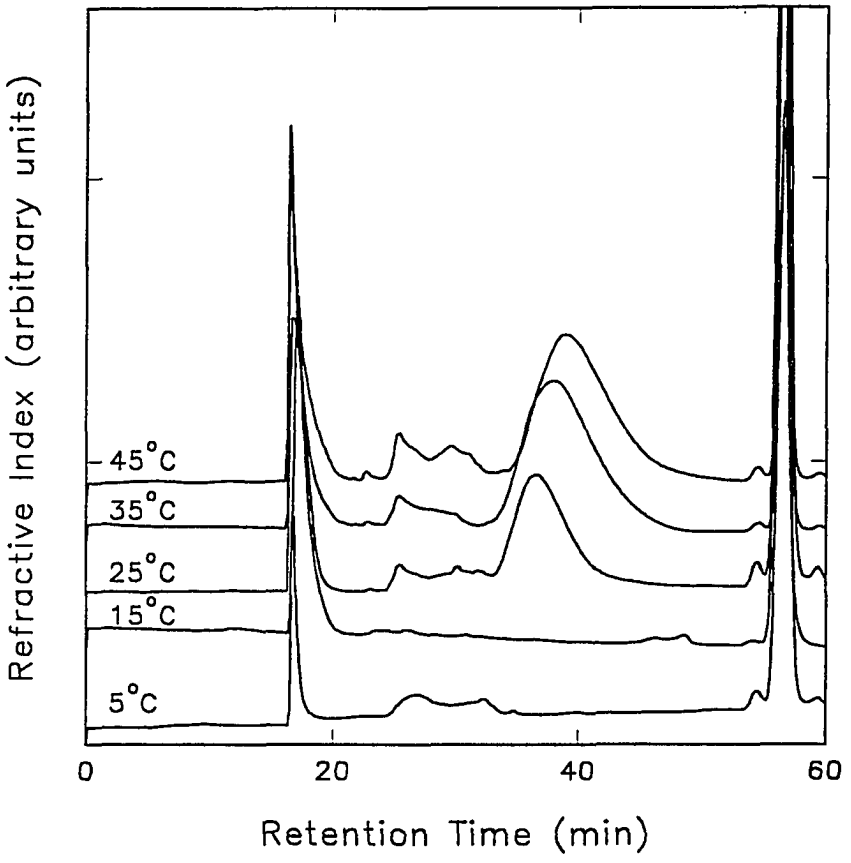


Figure 3. The chromatogram of retrograded amylose supernatant from different incubation temperature. The original amylose solution was prepared with water dispersion procedure with concentration 3.5 mg/mL and incubated at different temperature for 100 days.

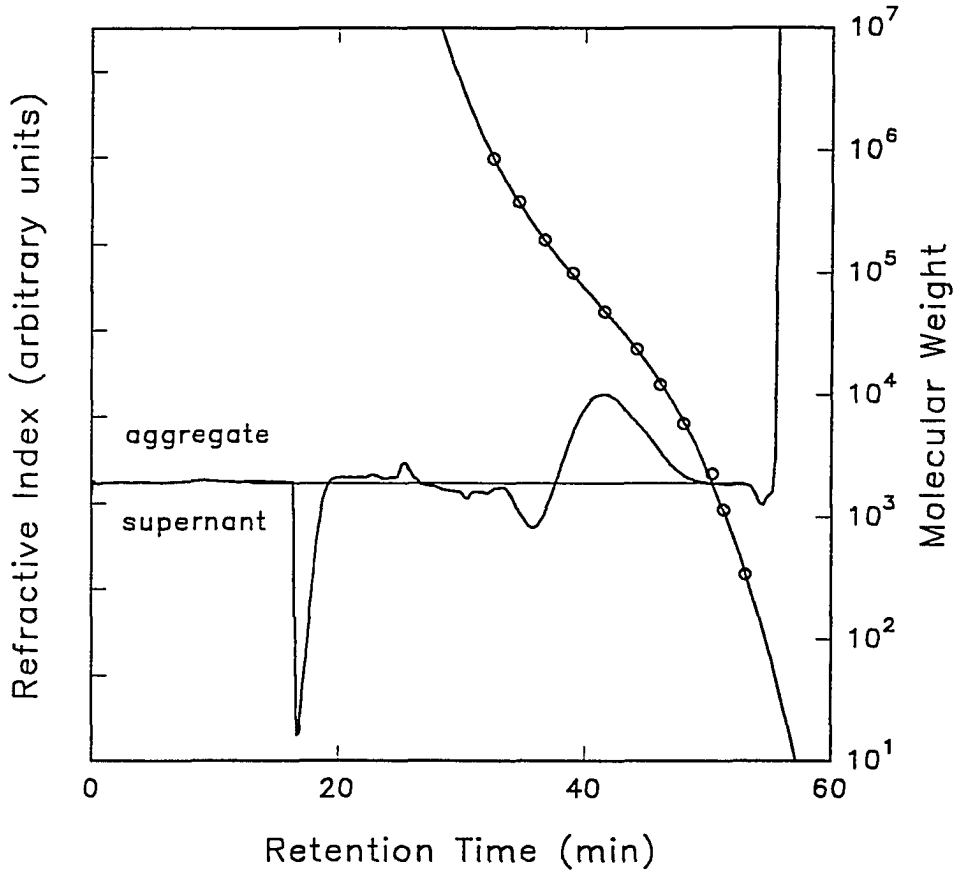
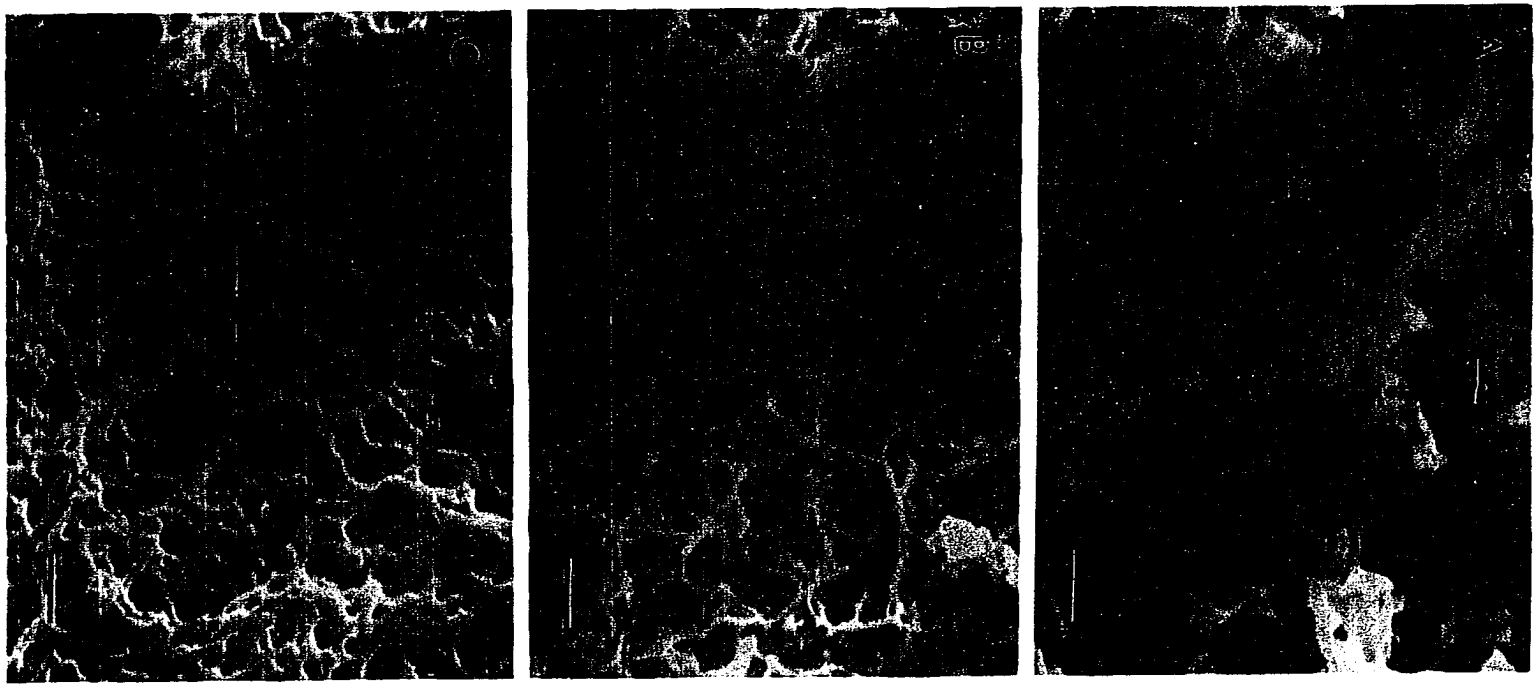


Figure 4. The difference chromatogram of original potato amylose and retrograded supernatant. The amylose solution was prepared with water dispersion procedure with concentration 3.5 mg/mL and incubated at 25°C for 100 days.



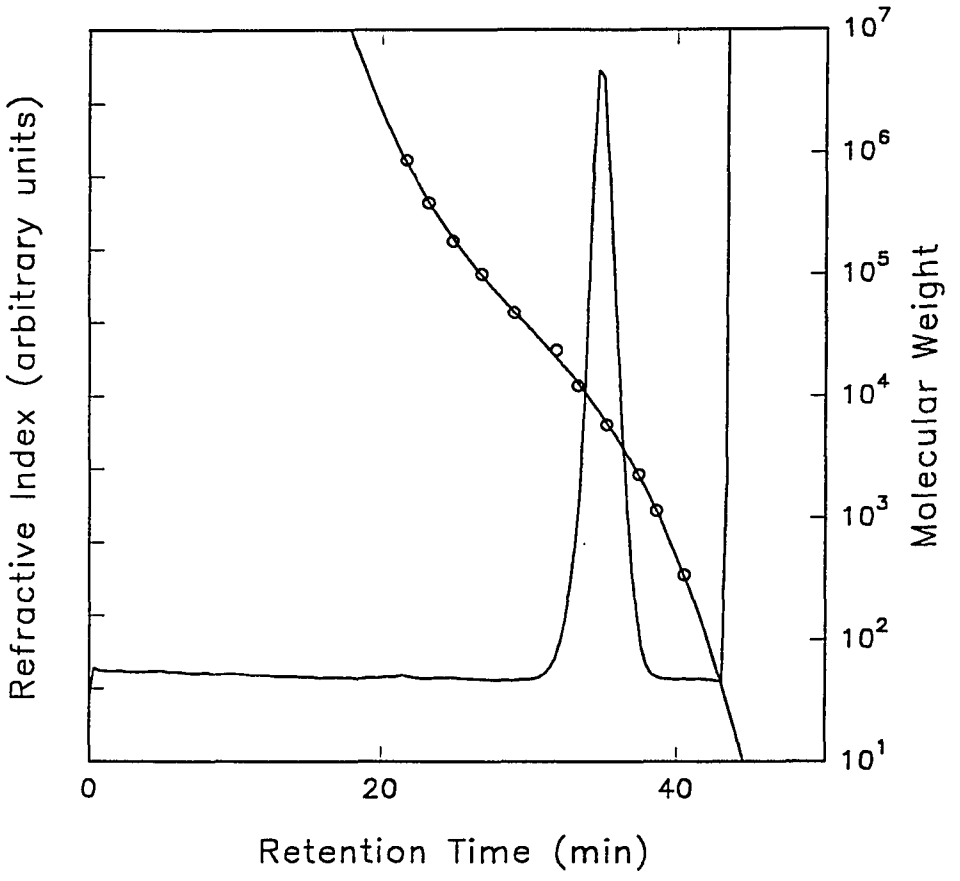


Figure 6. The chromatogram of retrograded amylose crystallite hydrolyzed with porcine pancreatic α-amylase. The -o- was molecular calibration curve of pullulan standards. The original amylose solution was prepared with water dispersion procedure with concentration 3.5 mg/mL and incubated at 5°C for 85 days.

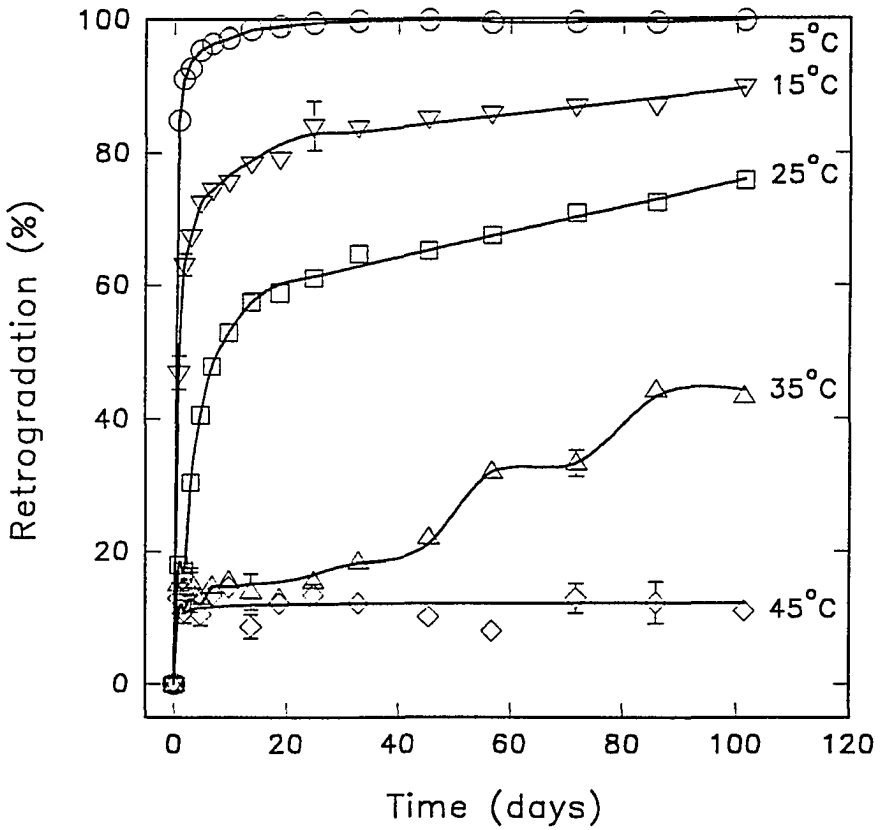


Figure 7a. The time course of 10.8 mg/mL amylose solution retrogradation. The amylose solutions were prepared with potassium hydroxide solubilization procedure.

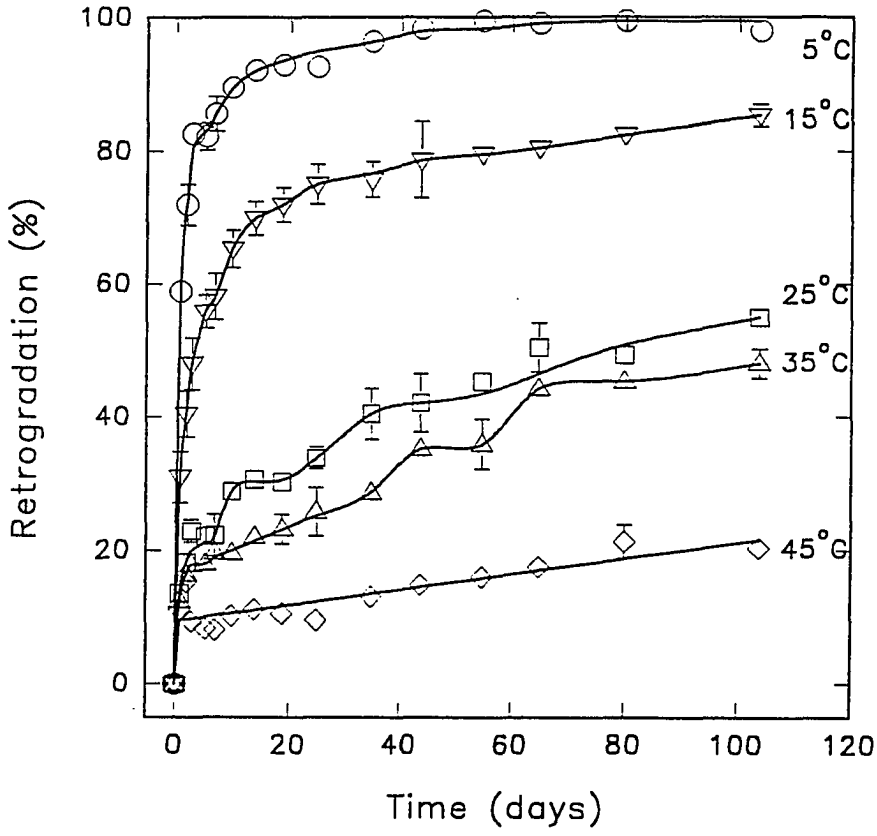


Figure 7b. The time course of 7.0 mg/mL amylose solution retrogradation. The amylose solutions were prepared with potassium hydroxide solubilization procedure.

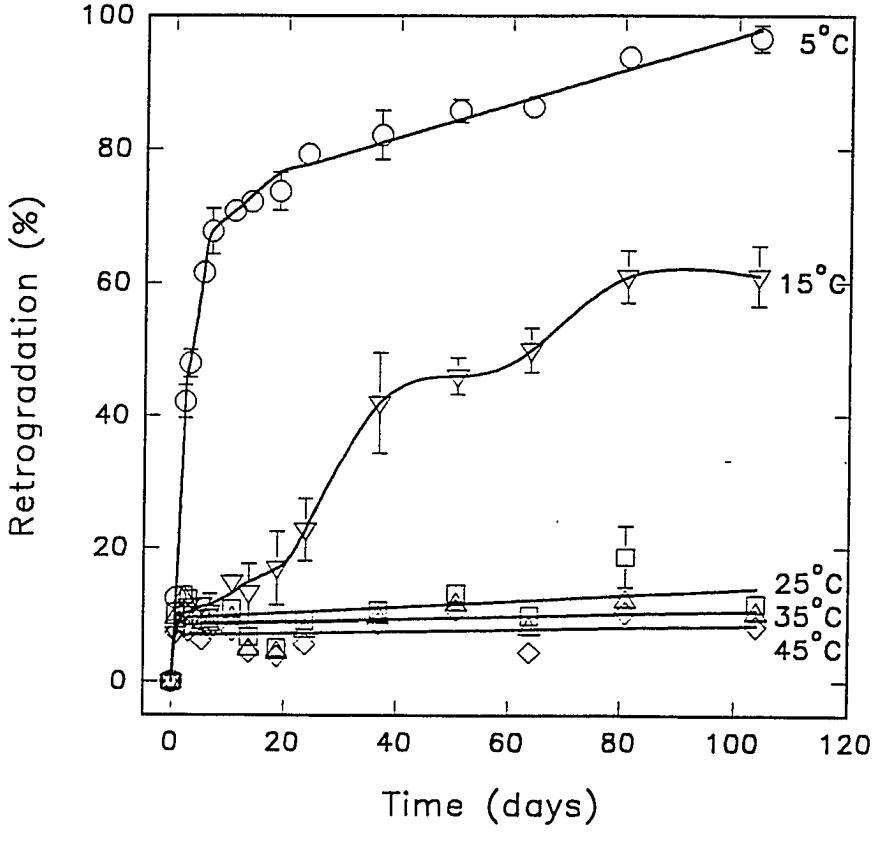


Figure 7c. The time course of 4.1 mg/mL amylose solution retrogradation. The amylose solutions were prepared with potassium hydroxide solubilization procedure.

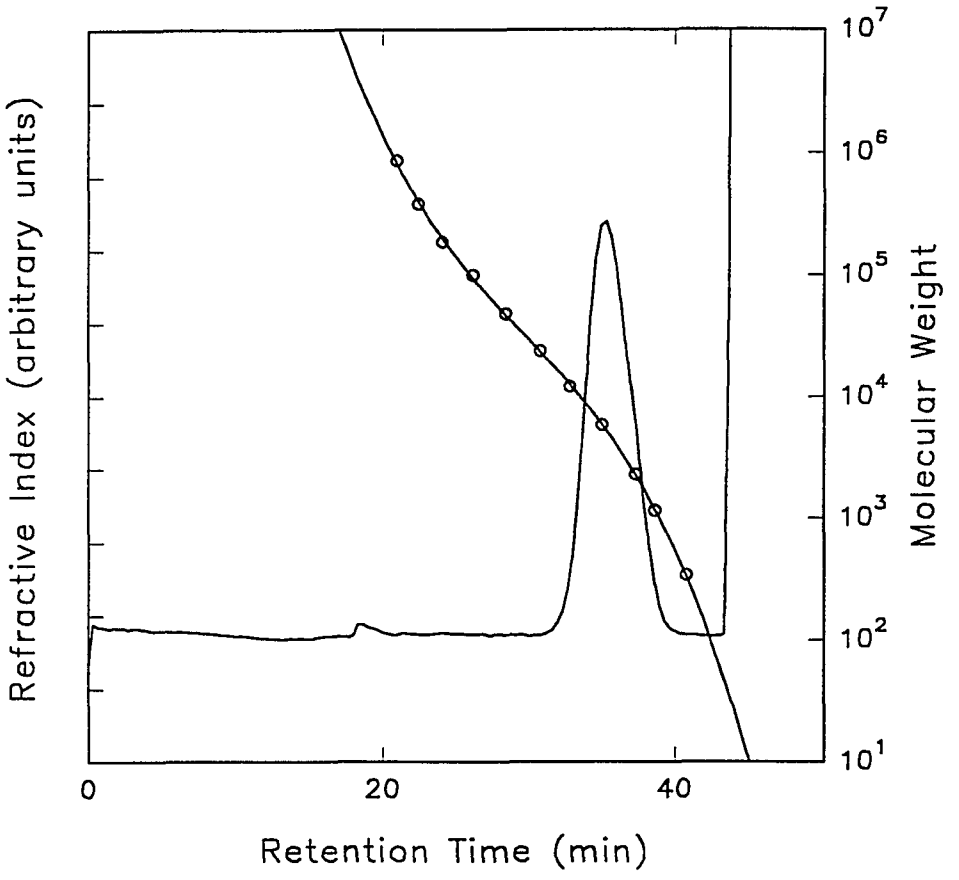


Figure 8. Chromatogram of retrograde amylose crystallite hydrolyzed with acid hydrolysis. The -o- was molecular calibration curve of pullulan standards. The original amylose solution was prepared with potassium hydroxide solubilization procedure with concentration 4.1 mg/mL and incubated at 5°C for 104 days.

GENERAL CONCLUSIONS

High performance anion-exchange chromatography with pulsed amperometric detection made it possible to distinguish minor starch structural changes. It was shown that a sodium nitrate gradient performed more efficient separations of amylodextrin than the sodium acetate gradient. The improved technique has become an important tool for carbohydrate analysis, though quantitative analysis still needs improvement.

Two genetically unrelated normal dent maize inbreds, ICI63 and ICI92, with different heterotic backgrounds responded differently to a developmental temperature change from 25 to 35°C. At 35°C, kernel weight and kernel density of both inbreds decreased, and ICI63 maintained the grain yield better than did ICI92. Increased grain developmental temperature affected starch morphology, chemical structure, and properties, such as increased small granule numbers and increased gelatinization onset temperature, as well as decreased amylose content. As developmental temperature increased, ICI63 amylopectin had an increased medium branch-chain fraction and decreased long and short branch-chain fractions, whereas, ICI92 had increased long and medium branch-chain fractions and a decreased short branch-chain fraction.

The *in vitro* model study elucidated temperature effects on the substrates of branching enzymes for maize starch synthesis. The results implied the complicity of starch biosynthesis and supported the hypothesis of branch formation mechanism of amylopectin proposed by Borovsky et al. (1975 and 1976). The hypothesis suggested that an intermolecule or intramolecule double helix conformation was required for

transferring branches by the branching enzymes. The retrogradation rate, an index of association and double helix formation in amylose molecules, decreased as a result of the increased temperature. The chain length of double helix segment of amylose increased as temperature increased. This study demonstrated that environmental temperature has an effect on the maize starch fine structure. In addition to temperature effects on starch synthesis enzymes, the simple physical phenomenon of growth temperature can directly affect the conformation of starch molecules.

APPENDIX

The purpose of this work was to respond the following requests from my committee:

1. Describe in more detail the number-average degree of polymerization (DP_n) determination that I used in my dissertation.
2. Give references to those papers which have used the method of size exclusion chromatography to determine polysaccharide DP_n.
3. Compare the value obtained from the method of size exclusion chromatography with the value from chemical analysis.

Request 1. Describe in more detail the number-average degree of polymerization (DP_n) determination that I used in my dissertation.

Materials. Potato amylose (Type III) and porcine pancreatic α-amylase were purchased from Sigma Chemical Co. (St. Louis, MO). Pullulan molecular weight standards (Shodex Standard P-82 kit, Showa Denko K.K., Tokyo, Japan) were purchased from Millipore Waters (Mildord, MA). The molecular weights and polydispersities of the standards were: 853,000, 1.14; 380,000, 1.12; 186,000, 1.13; 100,000, 1.10; 48,000, 1.09; 23,700, 1.07; 12,200, 1.06; and 5,800, 1.07. Maltotetradecaose was purchased form Nakano Vinegar Co. (Aichi, Japan) and maltoheptaose and maltose were purchased from Aldrich Chemical Co. (Milwaukee, WI). Maltotetradecaose,

maltoheptaose and maltose were used as oligosaccharide standards. Other chemicals were reagent grade, purchased from Fisher Scientific (Springfield, NJ) and used without further treatments.

Molecular weight of potato amylose. Molecular weight of potato amylose was determined by using a chemical analytical method and HPLC-size exclusion chromatography (SEC).

A. Chemical analytical method:

The procedure described by Hizukuri et al.[1,2] was followed for determining the degree of polymerization of amylose.

B. HPLC-SEC method:

Potato amylose was dispersed in 90% dimethyl sulfoxide and heated in a boiling water bath with stirring for 20 min and then stirred at room temperature overnight before being injected into the chromatographic system. An HP 1050 Series Pump System, equipped with a 20 μl sample loop and an HP 1047A Refractive Index Detector (Hewlett Packard, Wilmington, DE), was used for the analysis. An Ultra-Ware Integrated HPLC Mobile Phase Handling System (Vineland, NJ) was employed to filter, sparge, and pressurize the eluent with helium. The mobile phase was deionized water from a Milli-Q water system (Millipore Co., Bedford, MA) which had been filtered through a 0.2 μm nylon membrane. Potato amylose was analyzed by three sequentially connected columns of TSK-GEL (G6000PWXL, G4000PWXL and G3000PWXL (300 x 7.8 mm)) with a PWXL Guardcolumn (40 x 6 mm) (Tosohaas, Montgomeryville, PA) at 80°C and a 0.6

mL/min flow rate. The refractive index detector was maintained at 50°C. Chromatographic data were collected and processed on a NEC computer with Maxima 820 gel permeation chromatographic software (Millipore, Waters Chromatography Div., Milford, MA). The narrow standard calibration[3, 4] was performed by using pullulan and malto-oligosaccharide standards. A cubic standard curve was calculated using log molecular weight versus standard retention time. For the determination of the molecular weight averages, the chromatogram was divided into a number of slices and the molecular weight of each slice was determined by the calibration curve. The slice interval was set at 10 sec and the molecular weight distribution in each slice was assumed to be monodisperse. The number-average and weight-average molecular weights (M_n and M_w) were calculated by the following definition[4-11]:

$$M_w = \frac{\sum (A_i \times M_i)}{\sum A_i} \qquad M_n = \frac{\sum A_i}{\sum (A_i / M_i)}$$

In the equations, A_i is the area of slice i and M_i is the molecular weight of slice i. Degree of polymerization (DP) was calculated as molecular weight divided by 162.

Polydispersity (D), used to describe the molecular weight distribution of the polysaccharides, was defined as:

$$D = \frac{M_w}{M_n}$$

Request 2. Give references to those papers which have used the method of size-exclusion chromatography to determine polysaccharide DP_n.

Size-exclusion chromatography has been utilized in polysaccharide analysis for decades[5,12]. Using same principles of separation, HPLC-SEC has shown a higher efficiency and has become an important analytical tool for a wide range of polymers and polysaccharides[3, 4, 8-11, 13, 14]. The concepts and calculations of the averages of molecular weights for polymers[6-11] are aslo applied in the study of polysaccharides[5, 8]. In Table 1, there are a number of publications in which HPLC-SEC was used to determine the molecular weights of polysaccharides. The references are listed at the end of this appendix.

Table 1. References for Determination[1] of Polysaccharide Molecular Weight by Using the Method of HPLC-SEC.

Polymer	Calibration and Detection	Average Molecular Weight Reported	References
Rice amylose/ debranched amylopectin	Pullulan & MALLS	M_n, M_w, D	15
Amylose	pullulan	DP_n, DP_w, D	16
Amylose/ debranched amylopectin	Pullulan; MALLS	DP_n, DP_w, D	17
Pullulan	MALLS	M_n, M_w, D	18
Amylose/ debranched amylopectin	LALLS	DP_w CL_w	19

(*continued*)

Table 1. (*continued*)

Polymer	Calibration and Detection	Average Molecular Weight Reported	References
Carrageenan	MALLS	M_w	20
Debranched starch	pullulan	M_n	21
chitosan	MALLS	M_n, M_w, D	22
Amylose/amylopectin	pullulan	M_n, M_w (pullulan equivalent)	23
Pectin	pullulan/dextran	M_n, M_w, D	24
Mold polysaccharides	dextran	MW	25
Starch	dextran	MW	26
Barley starch	dextran	M_n, M_w, M_z, D	27
Starch	pullulan	Apparent MW	28
Debranched amylopectin	LALLS	CL	29
Debranched amylopectin	LALLS; (pullulan for instrumental constant)	CL,M_n, M_w, D	30
Starch	Dextran	M_w	31
Amylose	LALLS (pullulan for instrumental constant)	M_w	32
Starch	dextran	MW	33
Starch	dextran	MW	34

[1] Abbreviations used in this table: M_n, M_w, M_z: number-, weight-, and z-average molecular weight; MW: molecular weight; DP: degree of polymerization; CL: chain length; D: polydispersity; MALLS: multiple-angle laser-light scattering; LALLS: low-angle laser-light scattering.

Request 3. Compare the value obtained from the method of size-exclusion chromatography with the value from chemical analysis.

To compare results of chemical analysis and those obtained form HPLC-SEC method, potato amylose (purchased recently from Sigma Chemical Co. with different lot number to the one used in my dissertation research) and amylodextrin (obtained from enzyme hydrolysis of retrograded amylose crystallite prepared from amylose solution, 3.5 mg/mL incubated at 5°C) were analyzed. The results from the different analytical methods are shown in Table II. DP_n of the potato amylose measured by using HPLC-SEC method was 690. The DP_n of potato amylose measured by using modified Park-Johnson method (chemical analysis) was 1040 and was about 50% larger than the one obtained from SEC. There are reasons for this difference. First, different methods measure different properties of the amylose molecule to obtain its DP. The chemical analysis measures the concentration of glucose anhydride units and the concentration of reducing ends to calculate the DP of amylose. The HPLC-SEC method measures hydrodynamic volume of the amylose molecule, and the DP was determined by comparing the molecular size to those of the known molecular weight standards. Second, pullulan of the same DP has a larger hydrodynamic volume than amylose because amylose has a slightly branched structure and a random coil conformation which results in a less extended molecule[1, 32]. It was reported that the molecular weights of amylose specimens were about 35% higher than those of the pullulan specimens at the same

elution volume of HPLC-SEC[32]. The discrepancy of the difference may vary with the amylose specimens.

However, the values of DP_n's of amylodextrin measured by using the two methods were in good agreement. The DP_n's of the amylodextrin were 37 and 39 from the modified Park-Johnson method and the HPLC-SEC, respectively. The difference was not statistically significant. The results indicated the difference in hydrodynamic volume of amylodextrin and pullulan is negligible because of the small molecular size and lack of branch points on the amylodextrin produced in this study.

HPLC-SEC is a useful analytical tool for separating polysaccharides of different molecular size and determining their molecular weights and distribution. One major weakness of the method is the unavailability of amylose and amylopectin standards with narrowly distributed molecular weights. Pullulan, an analogous standard, has been chosen for this purpose[16, 17, 21, 23, 24, 28, 30, 32] and used as a standard for water-soluble polysaccharide analysis[35]. Using pullulan or dextran, standards currently available, results in errors in the determination of amylose and amylopectin molecular weights. Therefore, it is necessary to specify the standards used for calibration of the SEC profile. For accurately determining amylose and amylopectin molecular weights, it was suggested multiple methods or a molecular-weight sensitive detector[4, 17, 30, 36], e. g. MALLS, should be used.

Table 2. Number-Average Degree of Polymerization of Amylose and Amylodextrin Determined by Using Chemical Analysis and Size-Exclusion Chromatography[1].

Sample	$DP_{n,PJ}$[2]	$DP_{n,SEC}$[3]
Potato amylose	1040±60	690±30
Amylodextrin[4]	37±3	39±2

[1] Means and standard deviations of triple determinations.
[2] From modified Park-Johnson method.
[3] From HPLC-SEC.
[4] Retrograded amylose crystallite obtained by using porcine pancreatic α-amylase hydrolysis.

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