STRUCTURAL CHANGES INDUCED IN WAXY MAIZE STARCH AND NORMAL
WHEAT STARCH BY MALTOGENIC AMYLASES

by

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Abstract

Maltogenic amylases are widely being used as an antistaling agent in baking industry. However, their action on starch in granular, swelled and dispersed forms, important components formed during bread baking, is largely unknown. Actions of two maltogenic amylases- A and -B on waxy maize starch (WMS) (100% amylopectin) and normal wheat starch (NWS) (~25% amylose) were studied and compared. For any given starch type, starch form, and hydrolysis time, maltogenic amylase-B hydrolyzed both starches more than maltogenic amylase-A as seen through sugar profile analysis indicating its higher degree of multiple attack action (DMA). Their action on non reducing ends blocked compound, p nitrophenol maltoheptaoside, confirmed their endo action. Maltogenic amylase-B showed a higher endo to total enzyme activity ratio than maltogenic amylase-A at any given enzyme weight. Greater MW reduction of dispersed starches by maltogenic amylase-B indicates its higher level of inner chain attack (LICA). Interestingly, MW distributions profiles of swelled starch hydrolysates did not show significant differences irrespective of swelling temperatures. Both enzymes showed differences in oligosaccharides compositions in dispersed and swelled starches' reaction mixtures with sugars of degree of polymerization (DP) > 2 being degraded to glucose and maltose during later stages. For granular starches, enzymes followed a random pattern of formation and degradation of sugars with DP >2. MW distributions of hydrolyzed granular starches did not show significant shift until at the end of 24h when a low MW peak was observed. Morphological study of granular starches showed that maltogenic amylase-A mainly caused pinholes on WMS while maltogenic amylase-B caused surface corrosion with fewer pinholes. For NWS, both enzymes degraded A granules with deep cavities formation during later stages. A decrease in crystallinity of granular starches means that enzymes were able to hydrolyze both amorphous and crystalline regions. These results indicate that maltogenic amylase-B with a high LICA and high DMA possesses a better starch binding domain which can decrease the starch MW without affecting bread resilience.

Strucuture of maltogenic amylase-A modified amylopectin (AP) in relation to its retrogradation was also studied. AP retrogradation was completely inhibited at % DH $\geq$ 20. MW and chain length distributions of debranched residual AP indicated with increase in % DH, a high proportion of unit chains with DP $\leq$ 9 and low proportion of unit chains with DP $\geq$ 17 were

formed. Higher proportion of short outer AP chains which cannot participate in double helices formation supports the decrease and eventually complete inhibition of retrogradation. Thus, maltogenic amylase-A can play a very powerful role in inhibiting starch retrogradation even at limited DH (%).

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Dedication

This thesis is dedicated to my parents.

Chapter 1 - Introduction

Maltogenic amylases are widely being used in the baking industry as an antistaling agent. In the past, classification of several other amylases has been done based on their actions on starch. However, the action pattern of maltogenic amylases is still largely unknown. While some studies reported the endo action of this enzyme others evidenced its exo action. Some of the distinguishing features of this enzyme from endo acting enzymes are its maltose specificity, preferential hydrolysis of cyclomaltodextrins over starch, slower drop in potassium-iodide binding with increase in reducing value during earlier stages of amylopectin hydrolysis and its ability to cleave α-amyalse inhibitor, acarbose. Endo action of this enzyme was supported by large reduction in amylose molecular weight, location of its active site in an open cleft and its ability to cleave non reducing ends blocked compound, InDP5.

Starch which is an important component in bread undergoes several structural changes during bread baking. During bread making, starch which is initially present in its granular or native form, takes up water and swells upon application of heat. A part of the starch gets gelatinized, however, most of the granules are largely in their granular or swelled forms. When the bread is allowed to cool after the baking process, the amylose fraction of starch recrystallizes within few hours whereas long term firmness of bread is due amylopectin retrogradation. For this study, maltogenic amylases -A and -B were used to hydrolyze waxy maize and normal wheat starches in their granular, swelled and dispersed forms to examine the differences in their actions. The information gained will be helpful in order to understand the behavior of these enzymes and to guide their use in baking. Finally, gelatinized waxy maize starch was hydrolysed to different degrees and starch structural changes induced thereby were related with amylopectin recrystallization. Our main aim was to determine how much hydrolysis is required to prevent amylopectin from recrystallization.

Chapter 2 - Comparison of maltogenic amylases action on dispersed normal wheat and waxy maize starches

Enzymes are one of the tools we use to modify starch structure and render starch useful for various applications. Starch-hydrolyzing enzymes belonging to the glycoside hydrolase (GH) family are widely used in the textile, detergent, and baking industries (Kruger and Lineback, 1987). Normal starch comprises 75–80% amylopectin fraction, with the remainder amylose, whereas waxy starch comprises essentially all amylopectin (Hizukuri et al., 2006). The susceptibility of starch granules to enzyme action depends on several factors, including amylose and amylopectin content, particle size, starch crystalline structure, the presence of enzyme inhibitors, the degree of starch gelatinization, and the mode of action of the enzyme (Bijttebier et al., 2008; Bird et al., 2009).

Amylases have been classified in the past on the basis of their action pattern on starch (Goesaert et al., 2009b). Glucoamylases (EC 3.2.1.3) and β-amylases (EC 3.2.1.2) are exoenzymes, which cleave α-1, 4- glycosidic linkages, thereby releasing glucose and maltose, respectively, from non-reducing ends of starch. α-amylases (EC 3.2.1.1) are endoenzymes, which hydrolyse internal α- 1,4-glycosidic linkages of starch, glycogen, and other oligosaccharides. Pullalanases (EC 3.2.1.41) and isoamylases (EC 3.2.1.68) cleave α-1, 6-glycosidic linkages of starch (Kim et al., 1999; Robyt et al., 1967). Maltogenic α-amylase (glucan 1,4-α-glucanhydrolase, E.C. 3.2.1.133), an amylase from the *Bacillus* species that releases maltose as its main hydrolysis product (Christophersen et al., 1998; Outtrup and Norman, 1984), has a unique action pattern and is known as an antistaling agent (Duedahl Olesen, 1999; Sargent, 2008).

Various studies have been conducted to determine the action pattern of the maltogenic α-amylase. According to Bijjtebier et al. (2010), the active site structure of this enzyme, the number of subsites, and the position of catalytic residues in the active site determine productive substrate binding and the distribution of the resulting hydrolysis products. *Bacillus Stearothermophilus* maltogenic α-amylase Novamyl has five subsites in its active site that are involved in binding to glucose units. Like α-amylase (E.C.3.2.1.1), this enzyme belongs to GH family 13 and shares a similar amino acid sequence and catalytic domain with other enzymes in the family (MacGregor et al., 2001); however, in addition to having three domains typically

present in α-amylase (Jespersen et al., 1991) — A, a catalytic domain; B, involved in substrate binding; and C, responsible for enzyme activity — this enzyme has two additional domains, D and E (granular starch binding domain) (Maarel et al., 2002) like cyclodextrin glycosyltransferases (CGTases). The amino acid sequence of Novamyl's catalytic domains (A–C) was found to be approximately 50% identical to that of CGTases, approximately 20–28% identical to α-amylase (Dauter et al., 1999), and 40–86% identical to neopullalanases (E.C. 3.2.1.135) and cyclomaltodextrinases (E.C. 3.2.1.54). The N-terminal domain present in this enzyme, its preferential hydrolysis of cyclomaltodextrins over starch (Lee et al., 2002), and its ability to cleave the α-amylase inhibitor acarbose distinguishes it from a typical α-amylase (Kim et al., 1999). Christophersen et al. (1998) reported the endo action of this enzyme as shown by the large reduction in molecular weight of hydrolysed amylose and its action on a non-reducing end-blocked compound (InDP5). The location of its active site in an open cleft, which provides no structural barrier to cyclodextrins, and linear substrates further support its endo activity (Dauter et al., 1999). Bijttebier et al. (2010) found increased endo action of maltogenic enzymes on amylose with increasing temperature. Bowles (1996) and Kragh (2002) reported the exo action of this enzyme is supported by the release of maltose as the primary hydrolysis product with no requirement for non-reducing chain ends, but the released maltose was reported to be in the α-configuration (Dauter et al., 1999), unlike that released by exo enzymes. A slower drop in potassium iodide-iodine binding with increasing reducing value pointed to the exo action of maltogenic α-amylase during earlier stages of amylopectin hydrolysis (Goasaert et al., 2010). Maltogenic amylases are reported to be active at higher temperatures, and thus on gelatinized and enzyme-susceptible starches (Leman et al., 2006).

McCleary and Sturgeon (2002) and Duedahl Olesen et al. (2000) summarized all enzyme activity determination methods and categorized them into three classes: (i) reducing sugar assay/saccharogenic methods, (ii) chromogenic methods, and (iii) amyloclastic methods. Saccharogenic methods measure the number of reducing sugars formed during enzyme hydrolysis and include ferricyanide, Somogyi-Nelson, 3,5-dinitrosalicyil acid, and copper sulphate-biquinoline methods. Chromogenic methods employ colored substrates and include the Ceralpha method (AACC method 22-02-01), the Betamyl method, and the dyed starch method. Amyloclastic methods include iodometric and viscosity measurements after starch degradation by enzyme. None of these methods analyze the structure of the products created by hydrolysis.

Of all these methods, only the reducing sugars and Ceralpha methods meet the requirements of the Enzyme Commission of the International Union of Biochemistry (IUB) (McCleary and Sheehan, 1987; McCleary and Sturgeon, 2002). The Ceralpha method employs a non-reducing end-blocked compound, p-nitrophenol maltoheptaoside (BPNPG7), in the presence of excess quantities of a thermostable α-glucosidase. The Somogyi-Nelson method is based on the measurement of reducing ends produced upon hydrolysis of soluble starch. Compared with the Ceralpha method, which employs a short substrate, the Somogyi-Nelson method involves the use of a more complex starch structure.

During bread baking, starch gelatinization results in amylose leaching from the granules, which forms a network outside these granules while amylopectin expands into the granular space (Hoseney et al., 2008; Zobel and Kulp, 1996). During gelatinization, loss of crystalline order and swelling of granules occurs such that hydrogen bonds within crystallites are broken, which results in melting of crystallites. Starch gelatinization thus produces a hot suspension of porous, gelatinized, and swelled granules with an amylopectin skeleton suspended in an amylose solution (Colonna et al., 1992). Understanding enzyme action on the gelatinized starch network and the resulting changes in starch structure is important to understanding the roles of antistaling enzymes in baking. Limited endo action of α-amylase during bread baking produces fermentable and reducing sugars, which promote yeast fermentation for increased loaf volume and development of desired color and flavor via the Maillard reaction. Goesaert et al. (2009b) reported the limited endo activity of *Bacillus Stearothermophillus* maltogenic α-amylase contributed to weakening the starch network in bread, thus offering antistaling effects.

In this study, we first determined the endo activity (CU) and total enzyme activity (EU) of maltogenic amylase –A and maltogenic amylase-B using the Ceralpha method and the Somogyi Nelson method, respectively, and compared the ratio of CU to EU. Next, we studied and compared enzyme action on gelatinized waxy maize starch (WMS) and normal wheat starch (NWS). We chose these two starches to study the enzyme action on starch systems containing essentially all amylopectin and amylose and amylopectin, respectively, because both components play an important role in the final structure setting of bread. We followed the progress of starch hydrolysis by taking aliquots from the reaction mixture at different time intervals; we carried out MS distributions and sugar profile analyses to determine the hydrolysis products. Comparing the action of maltogenic amylases on two different starches gave better insight into the effects of

substrate accessibility by the enzymes, the extent of starch structure modification, and differences in carbohydrate compositions of the resulting starch hydrolysates. This information will help guide the use of these enzymes in baking applications.

Materials and Methods

Materials

WMS (Amioca) and NWS (Midsol 50) were obtained from National Starch LLC (Bridgewater, NJ) and MGP Ingredients Inc. (Atchison, KS), respectively. Moisture content was determined on the basis of weight loss at 130 °C for 120 min (AACC Method 44-15A) and was 10.2% for WMS and 10.6% for NWS. Maltogenic amylases-A and –B were provided by Caravan Ingredients (Lenexa, KS). All other chemicals and reagents used were of analytical grade.

CU determination

For CU determination, we followed AACC Method 22-02-01. We used enzyme solutions with differernet concentrations, but the substrate (p-nitrophenol maltoheptaoside) concentration was fixed at 5.45 mg/ml. The assay was performed at pH 5.0 and temperature 40 ⁰C. One CU is defined as the amount of enzyme in the presence of excess thermostable α-glucosidase required to release 1.0 µmol of p-nitrophenol from BPNPG7 in 1 min under defined assay conditions.

CU was calculated using following equation:

CU / g enzyme = (ΔE_{400}/ Incubation time) x (Total volume in cell / Aliquot assayed) x (1 /E_{mM}) x (Extraction volume / Sample weight) x Dilution,

where ΔE_{400} = Absorbance (reaction) - Absorbance (blank), incubation time = 10 min, E_{mM} of p-nitrophenol (at 400nm) in 1% tri-sodium phosphate = 18.1, extraction volume = 20mg/ml enzyme preparation, and Dilution = Dilution of original extract.

EU determination

We followed the Somogyi-Nelson method (Somogyi, 1952) for EU determination. Soluble starch (Mallinckrodt Chemical Works, St. Louis, NO) solution (1ml, 1% wt %) and enzyme solutions of different concentrations were incubated in separate tubes for 10 min at 40 ⁰C and 62 ⁰C for maltogenic amylase-A and 40 ⁰C and 55 ⁰C for maltogenic amylase-B. Enzyme

solutions (1ml) were added to each tube containing substrate solutions (1ml) and incubated for 30 min. At the end of 30 min, low-alkalinity copper reagent (1ml) was added and followed by heating at 100 ⁰C for 10 min. Arsenomolybdate reagent (1ml) was then added, and the volume was adjusted to 5 ml with distilled water. The solutions were kept at room temperature for 15 min before measuring absorbance at 500 nm against blank solution (without soluble starch).

Dispersed starch hydrolysis

Starch (1.8g db) was mixed with 90ml of 100mM sodium acetate buffer containing 5mM CaCl2 (pH 5.0), heated at 120 °C for about 40 min in a pressure bottle (Ace Glass Incorporated, Vineland, NJ), and cooled to optimum temperatures 62 °C and 55 °C for maltogenic amylase-A and maltogenic amylase-B, respectively. Enzymes (9EU/g starch db) were added to the starch slurries to initiate the enzyme reaction. This enzyme level selected was based on previous studies (Splender et al., 1999; Leman et al., 2009). Enzymatic starch hydrolysis was carried out for 4 h, taking aliquots after every 30 min and an aliquot at the end of 24 h. The enzymes were denatured in a boiling water bath for 10 min. Starch hydrolysates were cooled to 25 °C and centrifuged at 8,000g for 10min. A part of the supernatant was further analyzed for sugar profile analysis and the rest of the sample was freeze dried for further studies.

Sugar profile analysis by high performance anion-exchange chromatography (HPAEC)

Sugar profile analysis of the supernatants from WMS and NWS hydrolysates was carried out as previously described (Cai and Shi 2010). The eluents used were eluent A – 500 mM NaOH and eluent B - 150 mM NaOH with 0.5 M sodium acetate. The gradient program was as following: 85% eluent B (150 mM sodium hydroxide with 0.5 M sodium acetate) for 0-0.4min, 30% at 20min, 25% at 30min, 0% at 35 min, 0% at 40 min, 85% at 41 min, and 85% at 55 min. The concentration of the injected sample was 20 μg/ml in deionized water.

Molecular size distributions analysis by gel permeation chromatography (GPC)

MS distributions of starch hydrolysates were determined as previously described (Cai and Shi, 2010).

Statistical Analysis

Each experiment was performed in duplicate. Data were analyzed by analysis of variance (ANOVA) procedure with Tukey's studentized range (HSD) test using SPSS version 20.0 (IBM Corporation, Inc., Armonk, NY). Mean values from the duplicated experiments were reported. The least significant differences (LSD) for comparison of means were computed at $p < 0.05$.

Results

EU and CU determination

EU and CU at different enzyme concentrations for both enzymes are given in Table 1.1. Both enzymes exhibited endo activity on BPNPG7, and thus hydrolysed the compound into p-nitrophenol and free glucose in the presence of excess quantities of thermostable α-glucosidase, yielding a yellow color. At the same enzyme weight, maltogenic amylase- A and -B had somewhat similar CU values that continued to increase with increases in enzyme weight (Table 1.1). However, the ratio of CU (determined at 40 °C) to EU (determined at optimum enzyme activity temperature) was higher for maltogenic amylase-B than for maltogenic amylase-A at same enzyme weight (Figure 1.1). For EU determined at 40 °C, maltogenic amylase-B again showed a higher CU-to-EU ratio at any given enzyme weight than maltogenic amylase-A (Figure 1.1). This result indicates that maltogenic amylase-B had a greater endo action on the substrate than maltogenic amylase-A at equal enzyme weight, suggesting that maltogenic amylase-B can hydrolyse a greater number of bonds during its interaction with the substrate; in other words, it has higher degree of multiple attack action (DMA) (Robyt and French, 1967). To achieve the same EU levels (based on dry weight of starch) for starch hydrolysis, we added a higher weight of maltogenic amylase-B than maltogenic amylase-A, which indicates a higher endo activity for maltogenic amylase-B and a higher exo activity for maltogenic amylase-A at the same EU levels. Christophersen et al. (1998) reported that Novamyl was able to hydrolyse the substituted pentasaccharide indolehtyl-[5-(2-aminoethylamino)-1-naphthalnene-sulfonate]-6-deoxy-α-D-maltopentaoside (Indp5) as monitored by reduction in fluorescence, and hence concluded that Novamyl does not require an unblocked non-reducing end for its action. They attributed this result to the open groove structure of the substrate-binding surface of maltogenic enzyme, which can easily accommodate cyclodextrins and linear substrates (Dauter et al., 1999).

Sugar profile analysis

Although maltose was the major sugar produced by both enzymes, different oligosaccharide compositions were also found in reaction mixture for each enzyme (Tables 1.2 and 1.3). Maltogenic amylase-A produced sugars up to DP 6, whereas maltogenic amylase-B produced sugars up to DP 7 at the end of 30 min. These sugars were further degraded to glucose and maltose during the course of hydrolysis, with maltose the predominant sugar. For maltogenic amylase-A, sugars up to DP 2 and DP 4 remained in the reaction mixtures of NWS and WMS, respectively, at the end of 24 h. For maltogenic amylase-B, sugars up to DP 2 for NWS and sugars of DP 1, 2, and 4 remained in the reaction mixture of WMS at the end of 24 h. Both enzymes caused more than 50% starch hydrolysis, indicating inner chain hydrolysis, and the percentage of starch hydrolysis for WMS was higher than that of NWS at any given time. Percentage hydrolysis was higher for maltogenic amylase-B than maltogenic amylase-A for any given starch and hydrolysis time because of its higher endo activity. The ratio of maltose to glucose in the reaction mixture also was higher for NWS than WMS at any time, and this ratio continued to decrease with time. This ratio was higher for maltogenic amylase-A hydrolysed starches, indicating greater exo action for maltogenic amylase-A than maltogenic amylase-B. Previous studies (Bijttebier et al., 2010; Outtrup and Norman, 1984; Christophersen et al., 1998) also reported the maltose specificity of maltogenic α-amylase. Slaughter et al. (2001) concluded that the initial hydrolysis products formed from amylose containing starches are from amylose, which leaches out during gelatinization and/or from the starch chains of shorter length protruding from starch granules. This means that enzyme adsorption on granules is delayed at this point, and enzyme hydrolyses the solubilized subsrate first. For waxy starch, which contains essentially no amylose, Slaughter et al. (2001) reported that enzyme adsorption on the granules takes place during the first hydrolysis stage.

Molecular size distributions analysis

For both enzymes, the MS distributions of NWS and WMS hydrolysates showed a decrease in the high-MS fraction with a corresponding increase in intermediate- and low-MS fractions as hydrolysis time increased (Figures 1.2 and 1.3). At the end of 30 min, high-MS region already had shifted significantly. Such a drastic peak shift during the initial stages of hydrolysis indicates a high level of inner chain attack (LICA; ratio of increase in level of

reducing polysaccharides to the increase in total level of reducing sugars formed during AP hydrolysis, particularly during initial stages of hydrolysis), and thus an endo action; however, we observed limited peak shift as hydrolysis progressed. Maltogenic amylase -B caused a greater reduction in high-MS fraction than maltogenic amylase-A at any given time, probably due to its greater LICA and endo activity. This result indicates that for any given percentage of starch hydrolysis, maltogenic amylase-B hydrolysed a higher number of inner chain bonds than maltogenic amylase-A. At low DH (%; ≤ 5), we observed a limited peak shift for gelatinized WMS (5%) hydrolysed by maltogenic enzyme. This reflects its exo action (Goesaert et al., 2009a). On the other hand, α-amylases from *B. amyloliquefacians* and *A. orzyae* significantly reduced the amylopectin molecular size even at low DH (%) (Bijttebier et al., 2010), indicating their higher LICA (Goesaert et al., 2005).

Conclusions

The two maltogenic α-amylases altered the gelatinized starch structure in distinctly different ways, as noted in the differences in oligosaccharides composition released and MS distributions of hydrolysed products. MS distributions showed a greater shift to intermediate- and low-MS regions during the initial hydrolysis period, pointing to enzymes' endo action on starch; however, maltogenic amylase-B displayed greater endo action on starches than maltogenic amylase-A. During later hydrolysis stages, MS shifted gradually. Maltogenic amylase-B showed a higher CU-to-EU ratio at any given enzyme weight, which explains why this enzyme hydrolysed WMS and NWS to a greater extent than the other. Relating the amount of total reducing sugars released at any given hydrolysis time to CU indicated that maltogenic amylase-B had a higher DMA and LICA than maltogenic amylase-A. This higher endo action of maltogenic amylase-B on starches indicates that it could lead to greater weakening of the starch network, and that its action would result in limited starch dextrinization during early baking stages, thus improving final bread quality in terms of flavor, color, shelf life, and volume.

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Figure 2.1 Comparing endo enzyme activity (CU) determined at 40 °C and total enzyme activity (EU) determined at different temperatures for maltogenic amylases -A and -B
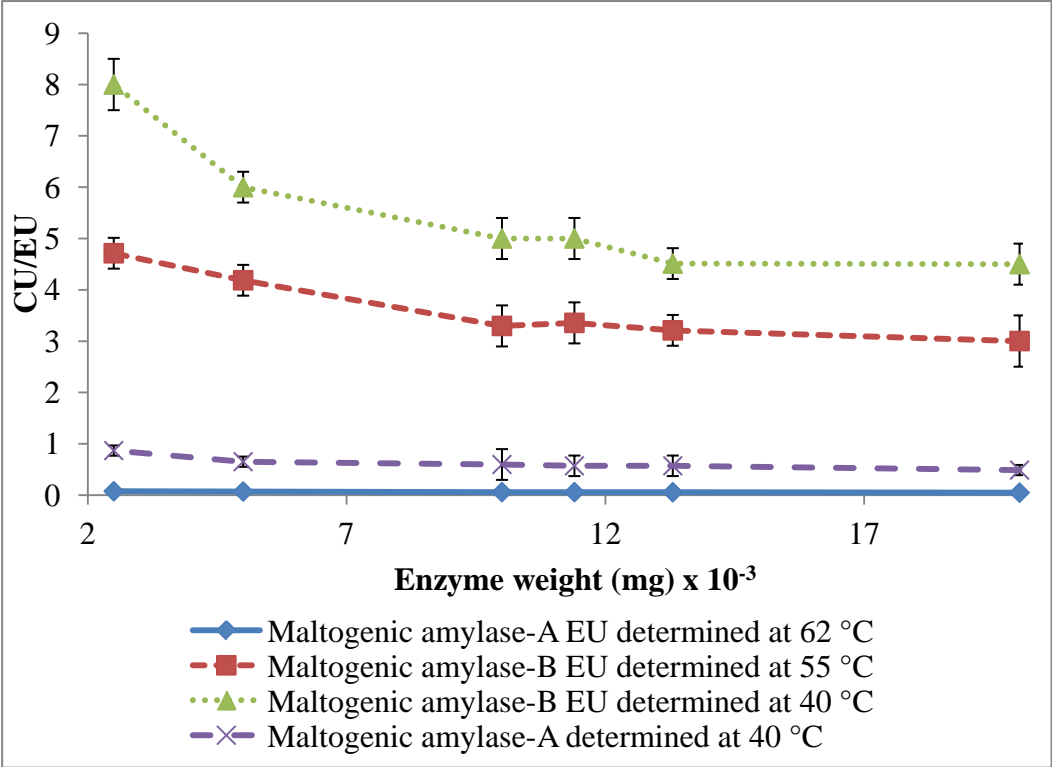
Figure 2.2 Molecular size distributions of dispersed starch hydrolysates produced by maltogenic amylase-B (a) normal wheat starch, (i) high and intermediate molecular size fractions and (ii) low molecular size fractions; (b) waxy maize starch, (i) high and intermediate molecular size fractions and (ii) low molecular size fractions

a
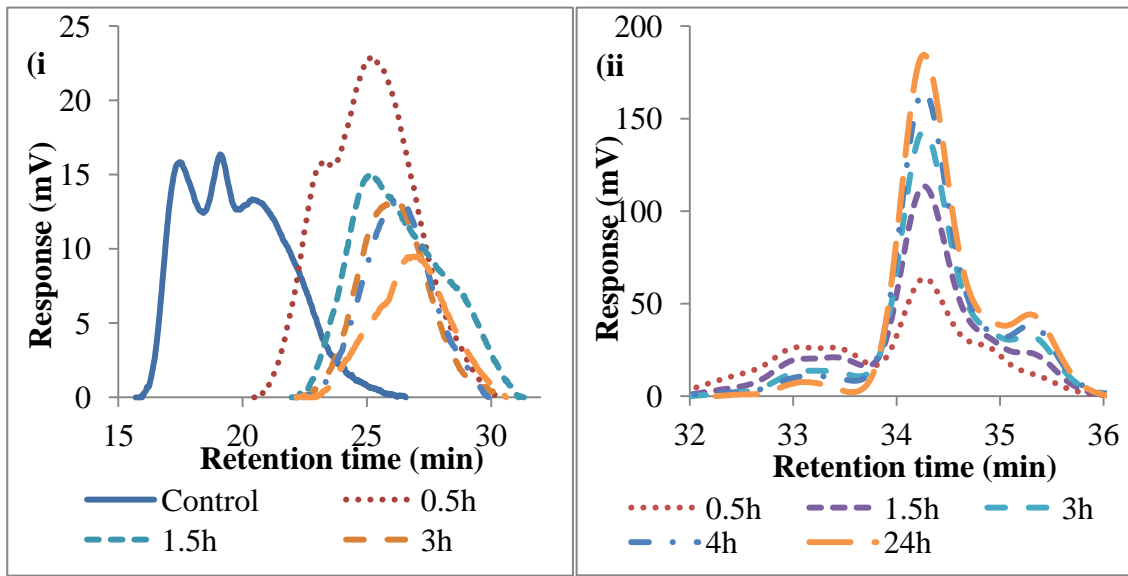
b
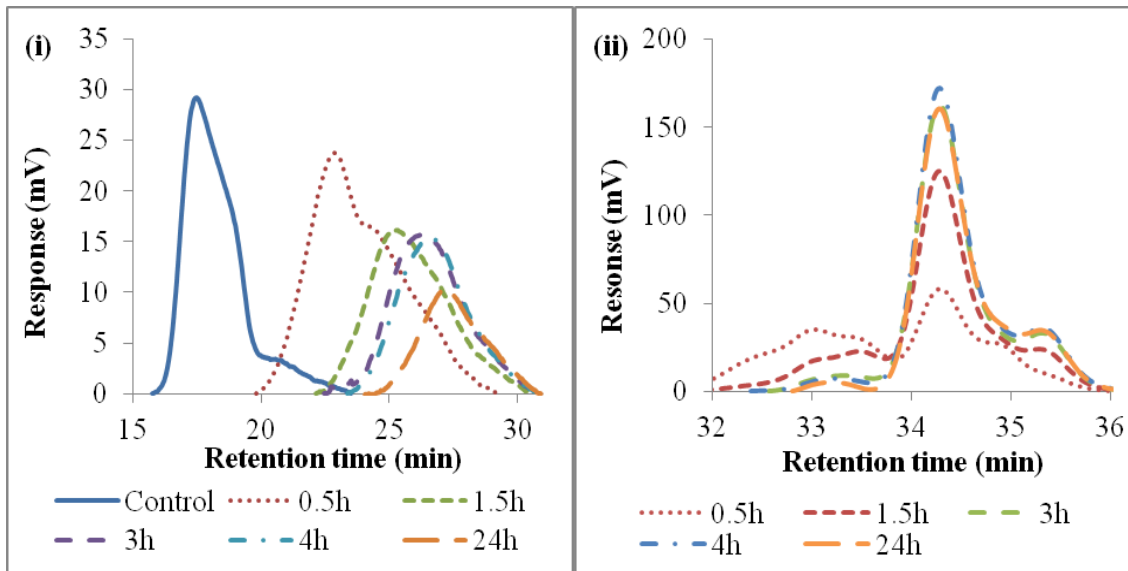
Figure 2.3 Molecular size distributions of dispersed starch hydrolysates produced by maltogenic amylase-A (a) normal wheat starch, (i) high and intermediate molecular size fractions and (ii) low molecular size fractions; (b) waxy maize starch, (i) high and intermediate molecular size fractions and (ii) low molecular size fractions

a
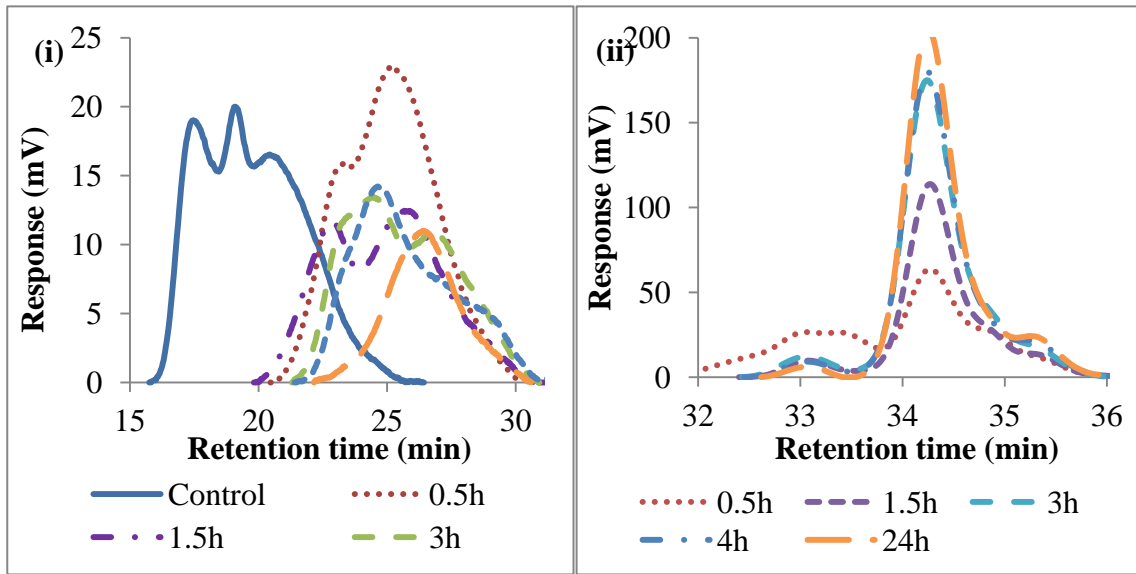
b
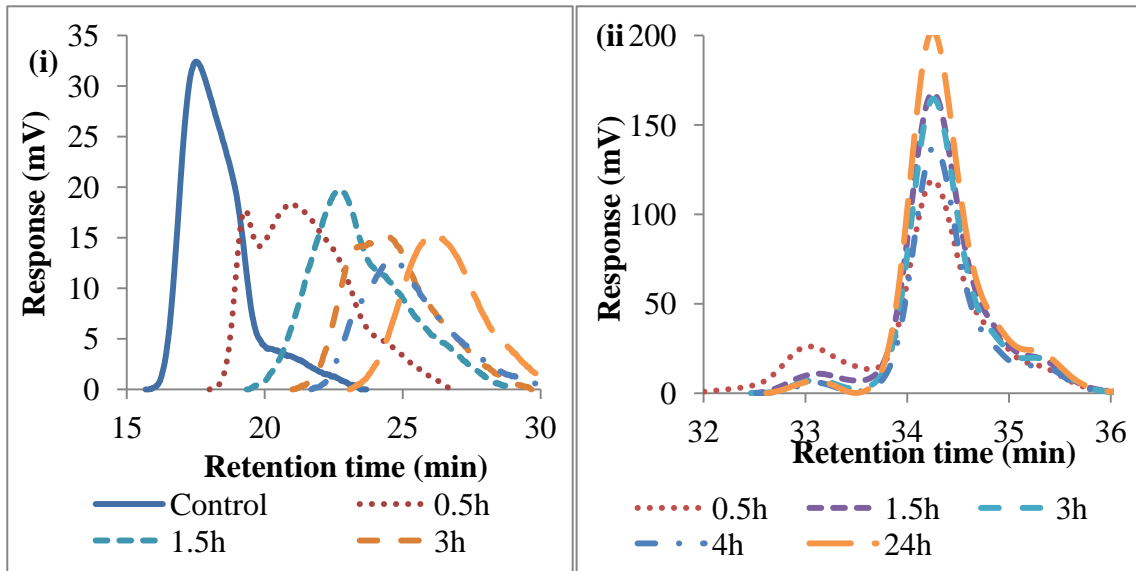
Table 2.1 Endo (CU) and total enzyme activity (EU) of maltogenic amylase-A and maltogenic amylase-B.

a. **Determined at 40 °C and 62 °C**

Maltogenic amylase-A				
Enzyme weight (mg) x (10^{-3})	CU/g enzyme	Ceralpha units (CU) x (10^{-3})	Enzyme units (EU) x (10^{-2}) determined at 40 ^{0}C	Enzyme units (EU) x (10^{-2}) determined at 62 ^{0}C
2.5	714.400	1.786	0.231	2.375
5.0	671.160	3.356	0.461	4.750
10.0	551.780	5.518	0.922	9.500
11.4	548.608	6.254	0.110	10.830
13.3	536.505	7.136	0.122	12.630
20.0	456.370	9.127	0.184	19.000

b.**Determined at 40°C and 55°C**

Maltogenic amylase-B				
Enzyme weight (mg) x (10^{-3})	CU/g enzyme	Ceralpha units (CU) x (10^{-3})	Enzyme units (EU) x (10^{-2}) determined at 40^{0}C	Enzyme units (EU) x (10^{-2}) determined at 55^{0}C
2.5	706.880	1.767	0.025	0.037
5.0	627.920	3.140	0.050	0.075
10.0	494.440	4.944	0.100	0.150
11.4	503.370	5.738	0.114	0.171
13.3	481.520	6.404	0.133	0.199
20.0	450.260	9.005	0.200	0.300

Table 2.2 Percentage of different sugars (based on initial weight of starch) in starch hydrolysates produced by maltogenic amylase - A

Hydrolysis time (h)	Waxy maize starch hydrolysates						% total sugars based on initial starch weight (db)[2]
	DP 1	DP 2	DP 3	DP 4	DP 5	DP 6	
0.5	0.4	30.6	1.6	11.6	4.4	0.6	49.6 ± 1.1^{e}
1.5	1.4	46.9	1.5	5.0	1.2	0.1	56.4 ± 0.5^{d}
3	2.1	54.9	0.5	1.1	0.0	0.0	58.9 ± 0.2^{cd}
4	4.0	60.9	0.2	0.3	0.0	0.0	65.5 ± 0.6^{b}
24	4.5	66.8	0.0	0.1	0.0	0.0	71.5 ± 0.8^{a}

Hydrolysis time (h)	Normal wheat starch hydrolysates						% total sugars based on initial starch weight (db)
	DP 1	DP 2	DP 3	DP 4	DP 5	DP 6	
0.5	0.3	34.4	5.7	5.0	1.0	0.7	47.4 ± 10^{e}
1.5	0.4	40.5	4.9	2.8	0.4	0.3	49.5 ± 1.0^{e}
3	0.6	44.4	2.2	2.6	0.1	0.2	50.0 ± 1.4^{d}
4	1.2	53.3	1.2	1.6	0.1	0.1	57.4 ± 1.9^{cd}
24	1.8	58.8	0.0	0.0	0.0	0.0	60.7 ± 0.3^{c}

[1]DP = degree of polymerization.

[2]Values with the same letter in the same column are not significantly different (p < 0.05).

Table 2.3 Percentage of different sugars (based on initial weight of starch) in starch hydrolysates produced by maltogenic amylase - B

Hydrolysis time (h)	Waxy maize starch hydrolysates							% total sugars based on initial starch weight (db)[2]
	DP[1] 1	DP 2	DP 3	DP 4	DP 5	DP 6	DP 7	
0.5	2.9	33.6	6.4	6.8	2.7	0.9	1.0	54.8 ± 0.4^e
1.5	5.2	45.5	4.2	4.5	0.9	0.0	0.0	60.4 ± 0.6^d
3	7.8	53.9	2.2	1.5	0.0	0.0	0.0	67.8 ± 0.4^{cd}
4	10.0	55.9	1.6	0.8	0.0	0.0	0.0	68.0 ± 1.2^b
24	11.2	61.2	0.0	2.0	0.0	0.0	0.0	73.8 ± 1.3^a
Hydrolysis time (h)	Normal wheat starch hydrolysates							% total sugars based on initial starch weight (db)
	DP 1	DP 2	DP 3	DP 4	DP 5	DP 6	DP 7	
0.5	2.1	33.1	5.3	6.8	1.8	0.9	1.0	51.1 ± 1.3^e
1.5	4.1	44.6	3.2	2.3	0.0	0.0	0.0	54.0 ± 1.1^e
3	4.6	51.0	0.5	0.8	0.0	0.0	0.0	56.9 ± 1.6^{de}
4	5.9	53.8	0.5	0.0	0.0	0.0	0.0	60.3 ± 0.6^{cd}
24	7.0	61.8	0.0	0.0	0.0	0.0	0.0	68.9 ± 0.6^c

[1]DP = degree of polymerization.

[2]Values with the same letter in the same column are not significantly different ($p < 0.05$).

Chapter 3 - Structural changes induced in granular and swelled waxy maize and normal wheat starches by maltogenic amylases

Starch is a mixture of two polysaccharides- amylose, which is a linear polymer and amylopectin, which is a branched polymer. The structure and properties of starch plays an important role in understanding enzyme action on starch (Oates 1997; Roder et al.2009). Enzyme action on starch depends on several factors such as granules porosity, integrity, crystallinity, phosphate content, proteins, lipids, and starch type, amylose to amylopectin ratio, molecular associations and gelatinization temperature (Buelon et al. 1998; Copeland et al. 2009; Gallant et al. 1992; Robertson et al. 2006; Wickramasinghe et ak, 2009; Zhang et al. 1999). Starch granules digestion involves several steps starting with diffusion of enzyme toward the substrate followed by enzyme adsorption on the starch and the hydrolytic event (Dona et al.2010; Lehmann and Robin 2007; Singh et al. 2010; Tester et al. 2006). Adsorption of amylase on the granule is a perquisite for starch hydrolysis (Slaughter et al. 2001). Factors such as total surface area and pores/crevices on starch structure wide enough to accommodate α-amylase molecule and the degree to which accessible glycosidic bonds in starch are exposed at the surface, determined the number of possible adsorption sites for the enzyme (Colonna et al. 1992). Adsorption of amylase on starch granules have been reported to be inhibited by oligosaccharides produced upon starch hydrolysis (Leloup et al. 1991). A significant correlation was found between degree of starch order and surface area with initial hydrolysis rate by α-amylase (Tahir et al. 2010).

Starch is semi crystalline in nature with growth rings of concentric semi crystalline shells (120-400nm thick) separated by amorphous regions. Semi crystalline shells consist of crystalline and amorphous lamellae with a repeating distance of 9-10nm thick. Native starch granules have 15-45% crystallinity as detected by X ray diffraction (XRD) and C NMR spectroscopy. In starch granules, amylopectin is responsible for crystallinity through ordered arrangements of double helices formed by adjacent branches within the structure (French 1984; Manners and Manners 1989). Depending on botanical origin, native starches display different diffraction patterns. A type pattern is shown by normal cereal starches while B type is shown by tuber and high amylose cereal starches (Buleon et al. 1998). Legume starches exhibit C type pattern due to the presence of both A and B type crystallites in their granules. For starches containing amylose-lipid complexes, a V type pattern is shown (Biliaderis 1992; Gerard et al. 2001). A type starches are

more susceptible to α-amylase hydrolysis than B type starches. Of A type starches, high amylopectin starches have higher susceptibility to hydrolysis by bacterial α-amylase (Leach and Schoch 1961) and α-amylase from *Bacillus subtilis* (Cone and Wolters 1990) than high amylose starches. Potato starch having the largest granule size and B type crystallinity has a reduced rate of hydrolysis (Noda et al. 2005; Planchot et al. 1997). Degree of crystallinity is another important factor influencing starch granule digestion with amorphous regions attacked preferably by alpha amylase (Gallant et al. 1992; Planchot et al. 1997). In another study (Zhang et al. 2006) both amorphous and crystalline regions were equally susceptible to α-amylase action.

Maltogenic α-amylase is widely being used in bread formulations as an antistaling agent. During baking, starch granules absorb water and swell in the presence of limited amount of water and are not completely gelatinized (Manners and Manners 1989) but remains in granular and swelled form. These two forms of starch thus form important components in bread. Action of maltogenic enzyme on dispersed starch molecules has already been studied (previous data). Significant molecular weight reduction during initial hydrolysis stages and a DH > 50 % of dispersed starches indicated its higher inner chain attack and thus an endo action. It also displayed endo action on reducing ends blocked compound (p nitrophenol maltoheptaoside). Starch gelatinization leads to complete disruption of molecular orders within the starch granule (Zobel 1984). Compared to gelatinized starch whose crystallinity has been lost, native starches are hydrolysed much slower due to the presence of double helices or amylose lipid complexes (Blazek and Gilbert 2010). Understanding maltogenic amylase action, which is widely being used as an antistaling agent in bakery foods, on granular and swelled forms becomes necessary in order to understand its behavior during baking.   The objective of this study was to study and compare the actions of maltogenic amylases on two different starches in native and swollen forms. To achieve this objective, samples were taken at different time intervals and analyzed for sugar released, molecular size distributions, morphological changes, gelatinization properties and crystallinity.

Materials and Methods

Materials

Waxy maize starch (WMS) (Amioca starch) and normal wheat starch (NWS) (Midsol 50) were obtained from National Starch LLC (Bridgewater, New Jersey) and MGP Ingredients Inc. (Atchison, Kansas), respectively. Moisture content was determined on the basis of weight loss at 130°C for 120 min. (AACC Method 44-15A) and was 10.2% for waxy maize starch and 10.6% for normal wheat starch. Maltogenic amylases- A and –B were provided by Caravan Ingredients (Lenexa, Kansas). Enzyme activity was assayed by quantifying the reducing sugars released from soluble starch (1.0% (w/v) solution) (St.Louis, MO) according to the Somogyi-Nelson method (Somogyi 1952) relative to a maltose standard curve. One enzyme unit (1EU) is the amount of enzyme that releases 1μmol of maltose/min at the optimum temperature as defined for each enzyme, 55°C for maltogenic amylase - B and 62°C for maltogenic amylase - A and pH 5.0 (100mM sodium acetate buffer containing 5mM $CaCl_2$). All other chemicals and reagents used were form Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

Swelled starch hydrolysis

Starch (1.8g db) was mixed with 90ml of 100mM sodium acetate buffer containing 5mM $CaCl_2$ (pH 5.0), swelled at different temperatures (70°C, 80°C, 90°C) for 30 min. in a pressure bottle (Ace Glass Incorporated, Vineland, New Jersey), and cooled to optimum temperatures 62°C and 55°C for maltogenic amylase- A and maltogenic amylase - B, respectively. Enzymes (9EU/g starch db) were added to the starch slurries to initiate the enzyme reaction. This enzyme level was selected based on previous studies (Leman et al. 2009; Splender et al. 1999). Both enzymes were added in amounts in order to be at same EU levels. Enzymatic starch hydrolysis was carried out for 4h, taking aliquots after every 30 min. and an aliquot at the end of 24h. Enzyme reaction was stopped by lowering pH 3.0 by adding 1M HCl, kept in ice-water bath for 30min and then neutralizing back to pH 6.5 by adding 1M NaOH, centrifuged at 8,000g for 10min. A part of the supernatant was further analyzed for high performance anion exchange chromatography (HPAEC) and the rest of the sample was freeze dried for further studies.

Granular starch hydrolysis

Similar procedure as mentioned above was used to hydrolyze granular starches except that starch slurries of NWS and WMS were kept at 40°C for 30min before adding enzyme (9 EU/g starch). Hydrolysis was then carried out at 40 °C. The rest was same as in the hydrolysis of swollen starch granules.

Sugar profile analysis by high performance anion-exchange chromatography (HPAEC)

Sugar profiles of the supernatants from WMS and NWS hydrolysates were analyzed according to the procedure cited earlier (Cai and Shi 2010a; Cai et al. 2010b). The gradient program was as following: 85% eluent B for 0-0.4min, 30% at 20min, 25% at 30min, 0% at 35min, 0% at 40min, 85% at 41min and 85% at 55min. The concentration of the injected sample was 20 μg/ml in deionized water. Degree of hydrolysis (%) (DH) was expressed as the total amount of sugars released at any given time based on initial dry weight of starch.

Molecular size distributions analysis by gel permeation chromatography (GPC)

Molecular size (MS) distributions of starch hydrolyzates were determined according to the procedure cited earlier (Cai and Shi 2010a; Cai et al. 2010b).

Light microscopy of swelled and granular starch hydrolysates

Samples were dispersed in water (10mg/ml) using a vortex mixer. A drop of sample suspension was transferred onto a slide covered by the coverslip. The sample was viewed with an Olympus BX 51 microscope (Olympus America Inc., Melville, NY). Images and photographs were captured under 40× magnification using SPOT Insight camera and SPOT 4.6 Windows software (Diagnostic Instrument Inc., Sterling Heights, MI, USA).

Scanning electron microscopy (SEM) of granular starch hdyrolysates

Granular starch hydrolysates were coated with gold palladium by using a sputter coater (Denton Vacuum, LLC, Moorestown, NJ) and viewed at 1000× and 5000x resolution with a scanning electron microscope (S-3500N, Hitachi Science Systems, Ltd., Japan) operating at an accelerating voltage of 20 kV.

Differential scanning calorimetry (DSC) of granular starch hydrolysates

Granular starch samples in water were prepared with starch: water ratio of 1:2. The DSC pans were sealed and analyzed in duplicates using a TA Q5000 (TA Instruments, New Castle, DE, USA). An empty pan was used as a reference. Samples were heated from 10 to 140 °C at 10 °C/min. The onset (To), peak (Tp), and conclusion (Tc) temperatures and enthalpy (ΔH) were obtained from the DSC endotherm with DSC software (TA Instruments, New Castle, DE, USA).

X-ray diffraction (XRD) of granular starch hydrolysates

X-ray diffraction of granular starches was conducted with a Philips X-ray diffractometer with Cu Ka radiation at 35 kV and 20 mA, a theta-compensating slit, and a diffracted beam monochromator. The moisture content of all the samples was adjusted to about 18% in a sealed dessicator at room temperature before analysis. The diffractograms were recorded between 2° and 35° (2θ).

Statistical Analysis

Each experiment was performed in duplicate. Data were analyzed by an analysis of variance (ANOVA) procedure with Tukey's studentized range (HSD) test using SPSS version 20.0 (IBM Corporation, Inc., Armonk, NY, USA). Mean values from the duplicated experiments were reported. The least significant differences (LSD) for comparison of means were computed at $P < 0.05$.

Results and Discussion

Sugar profile analysis of swelled starch hydrolysates

The progress of starch hydrolysis was monitored by measuring the oligosaccharides released at specific time intervals during reaction. Different oligosaccharides composition is given in Tables 2.3 to 2.8. For starches swelled at 90°C, maltogenic amylase-A released sugars up to DP-4 from WMS and sugars up to DP- 6 from NWS at the end of 30 min which was further hydrolysed to glucose and maltose by the end of 24h via secondary reactions. For starches swelled at 80°C, sugars up to DP- 6 and DP-4 were released at the end of 30 min for WMS and NWS, respectively. For both starches, although the concentration of DP-3 and DP-4 continued to decrease these sugars were still present in the reaction mixture along with glucose

and maltose at the end of 24h. Maltogenic amylase-A released sugars up to DP- 7 and DP-5 from WMS and NWS swelled at 70°C, respectively, at the 30min. Higher sugars up to DP-5and DP-4 were still present in the reaction mixture of WMS and NWS at the end of 24h, respectively. DP of sugars released at the end of half hour decreased with increase in swelling temperature or increase in substrate accessibility.

Maltogenic amylase - B hydrolysed WMS and NWS, swelled at 90°C, released sugars up to DP-7 at the end of 30 min. For both starches, sugars up to DP-3 were present in the reaction mixture at the end of 24h. For swelling done at 80°C, sugars up to DP-7 and DP-6 were released for WMS and NWS, respectively at the end of 30min which were hydrolysed to glucose and maltose via secondary reactions by the end of 24h. Sugars up to DP-7 were released at the end of 30 min for starches, swelled at 70°C which were further hydrolysed to glucose and maltose for WMS while for NWS DP-3 was also present at the end of 24h. At all swelling temperatures, maltogenic amylase- B released sugars up to DP – 7 at the end of half hour which degraded to sugars up to DP -2 at the end of 24h.

For all the swelled starch hydrolysates, maltose to glucose ratio was higher for WMS than NWS and the ratio decreased with increase in time as opposed to dispersed starch hydrolysates where this ratio was higher for NWS than WMS (previous data). Irrespective of the swelling temperature, maltogenic amylase -B resulted in a higher DH than maltogenic amylase-A for both starches at any hydrolysis time due to its higher degree of multiple attack (DMA) action and higher endo activity. Also different oligosaccharides compositions produced by both enzymes confirm their different DMA actions on starch.

Sugar profile analysis of granular starch hydrolysates

For granular starches, the granular integrity and crystallinity remains intact restricting the enzyme action on starch (Blazek et al. 2010; Lauro et al. 1993). Individual sugar compositions are given in Tables 2.1 and 2.2. Sugar profile analysis of maltogenic amylase - A hydrolysed starches, showed that during the initial hydrolysis time period, only DP-1 and DP-2 were produced for NWS while for WMS, DP-4 was also produced at the end of 30min. While the concentration of DP-1 and DP-2 continued to increase for both starches, higher DP sugars followed a pattern of degradation and accumulation during the course of hydrolysis. With an increase in hydrolysis time, enzyme was able to penetrate deep in granules thereby releasing

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higher DP sugars with time. Not a significant difference was observed in DH (%) for both starches except at the end of 4h and 24h, where DH (%) of NWS was higher than DH (%) of WMS.

Maltogenic amylase -B, however, behaved somewhat differently on granular starches than maltogenic amylase- A. At the end of 30 min, higher sugars up to DP-4 and DP-5 were already formed for WMS and NWS, respectively opposed to maltogenic amylase - A where higher sugars appeared during later hydrolysis stages. While sugars up to DP-4 continued to increase with time till the end of 4h, after which sugars with DP-3 and DP-4 gradually decreased. Maltogenic amylase -B caused a random cleavage of glycosidic bonds because at the end of half hour, DP-1, DP-2 and DP-4 were released for WMS while at the end of 24 h, it also produced sugars up to DP-5. For NWS, glucose and maltose were produced at the end of 30min but at the end of 24 h, sugars up to DP-4 were also produced in the reaction mixture. Similar DH (%) for both starches was observed except at the end of 24h, where DH (%) of NWS was higher than that of WMS. Irrespective of the starch type, maltogenic amylase - B action on granules resulted in a higher DH and different oligosaccharides compositions than that of maltogenic amylase - A. Very low DH (%) of granular starches even till the end of 24h, opposed to DH (%) = 80 observed for granular normal maize starches (5%) hydrolyzed by bacterial α-amylase from *Anoxybacillus flavothermus* (Tawil et al. 2012), indicates that maltogenic amylases are not very active on intact granules. Accessibility of inner parts of native starches greatly determines the hydrolysis rate (Tester et al. 2006). Carbohydrates released during initial stages are broken down first by the enzyme before it acts on the rest of the granule (Manelius et al. 1997). While the action of α-amylase on amylose was found to be a random one, action on amylopectin was non - random (Colonna et al. 1992).

Also a higher DH (%) observed for NWS than WMS during later stages is quite interesting because waxy maize starch have been shown to be more susceptible to enzymatic action (Buleon et al. 1998; Gallant et al. 1992; Planchot et al. 1997; Seneviratne and Biliaderis 1991). Longer incubation times of waxy maize, normal and high amylose starches with α-amylase resulted in higher degree of hydrolysis of waxy maize starch than normal and high amylose starches containing amylose-lipid complexes (Tester et al. 2004). Waxy maize starch with low resistant starch content (4.9 %) than that of wheat (5.6 %) is believed to be digested more easily by enzyme (Zhang et al. 2006). An inverse relationship between native amylose

containing starches and α-amylase digestion (Gallant et al. 1992; Zhang and Hamaker 2009) is attributed to the presence of amylose-lipid complexes which restricts enzyme action (Karkalas et al. 1992; Lauro et al. 1999). Similar conclusions were made where a greater susceptibility of high amylopectin starches than high amylose starches to bacterial α-amylase action (Leach and Schoch 1961) and *Bacillus subtilis* α-amylase (Cone and Wolters 1990). However, no direct correlation of crystallinity levels and amylose contents with final hydrolysis extents (48h) of maize mutant starches was reported (Gerard et al. 2001). This final hydrolysis extent was found to be correlated with the amount of B crystalline type.

Molecular size distributions of swelled starch hydrolysates

Figures 2.3 to 2.8 show the MS distributions of swelled WMS and NWS hydrolysates. A significant shift of high MS fraction was observed at the end of 30 min for all hydrolysates and this shift was higher for starches swelled at higher temperatures indicating increase in endo activity of the enzymes with greater starch susceptibility. Later on, this shift became more gradual with a concomitant increase in low MS fraction with time. This slower MS shift could be due to products inhibition or starch structural inhibition offered to the enzyme action. Maltogenic amylase - B being higher in endo activity caused a greater shift than maltogenic amylase - A during initial stages at any given time, temperature and starch type. Irrespective of the swelling temperature, maltogenic amylase - B hydrolyzed both AP and AM fractions in NWS to a greater extent than maltogenic amylase - A due to its higher level of inner chain attack (LICA). AP fraction was almost completely degraded for NWS and WMS hydrolyzed by both enzymes. At all swelling temperatures, the MS distributions showed similar profiles for WMS and NWS for each enzyme suggesting that their LICA remains independent of substrate accessibility.

Molecular weight distributions of granular starch hydrolysates

Novamyl hydrolysed granular waxy maize starches showed similar MW distributions to control native starch till the end of 4h (Figure 2.1 a). WMS hydrolysates showed two distinct peaks corresponding to high and intermediate MW fractions with a third tailing peak which became more apparent at the end of 24h along with an appearance of low MW peak. On the other hand, control native NWS showed a bimodal MW distributions corresponding to amylopectin and amylose fractions. Upon hydrolysis, a trimodal MW distribution was observed with a gradual decrease in high MW fraction and corresponding increase in intermediate MW

fraction. A low MW peak was noticed at the end of 24h (Figure 2.1 b). A nearly same trend of MW distribution followed by amyloglucosidase hydrolysed WMS starch compared to native WMS was attributed to its localized attack on granules (Brewer et al. 2012).

Starches hydrolyzed by maltogenic amylase - B showed a greater shift during initial hydrolysis stages than maltogenic amylase - A hydrolysed starches. Maltogenic amylase - B hydrolysed WMS starches also showed bimodal MW distributions with a low MW peak being formed at the end of 24h (Figure 2.2 a). A trimodal MW distribution was observed for hydrolysed NWS starches similar to that observed for maltogenic amylase -A hydrolysed NWS starches (Figure 2.2 b). Limited shift in MW distributions of native high amylose and corresponding starches hydrolysed by α-amylase, amyloglucosidase and combination of these two enzymes was suggested to be due to their heterogenous attack on granules (Brewer et al. 2012). Limited and nearly same MW distributions of hydrolysed starches compared to control, observed with our study, could also be due to the heterogenous and localized attack of maltogenic enzymes on starch granules.

Light microscopy of swelled and granular starch hydrolysates

Hydrolyzed WMS and NWS were examined under normal and polarized light microscopy to follow changes occurring in starch morphology as the hydrolysis progressed. Different patterns of morphological changes were observed when different starches were used. Starches hydrolysed in their granular form did not show any significant damage till the end of 4h with most of the granules exhibiting birefringence. For NWS, both enzymes produced concentric layers like structure of the granules at the end of 24h (Figure 2.9 b and 2.10 b). At the end of 24h, maltogenic amylase - B hydrolysed WMS, showed a greater damage to starch granules than maltogenic amylase - A (Figure 2.10). For starches swelled at 70℃, extensive starch granules damage was observed at the end of 4h and 24h while a small percentage of granules still exhibited birefringence (Figures 2.11 to 2.12). Morphology of maltogenic amylase -B hydrolysed NWS at the end of 30min and 4h, showed a greater one sided cleavage of most granules (Figure 2.12 b). Little or no birefringence was observed for starches swelled at higher temperatures (data not shown).

Scanning electron microscopy (SEM) of granular starch hydrolysates

SEM images for granular starch hydrolystes are shown in Figures 2.13 to 2.14. Maltogenic amylase - A action on WMS started with pinholes formation on few granules which widened up during later stages while majority of granules were found to be largely intact. This means that the enzyme first binds some specific sites on the granules and the degradation starts from this point with enzyme moving along a chain and eventually leading to formation of deeper grooves and channels via an endo action. Widening of pinholes due to endo action was previously reported (Aggarwal and Dollimore 1998; Shariffa et al. 2009). A similar action was noticed for alpha amylase from *Aspergillus fumigatus* on high amylose, normal maize and waxy maize starch granules. At the end of 24h, a swiss cheese appearance, typical of an alpha amylase action on starch, was observed (Planchot et al. 1995). Maltogenic amylase - B, on the other hand, formed fewer pinholes and showed more of surface erosion on WMS while keeping most of the granules intact. Relative susceptibility of different areas of native corn and wheat starch granules has been noticed by α-amylase action (Evers and Ee 1970; Evers et al. 1971). Compared to maltogenic enzymes action on waxy maize starch granules, amyloglucosidase action on corn starch granules result in wider pores distribution in surface patterns (Fannon et al. 1992).

NWS shows a bimodal granule size distribution comprising of A-granules (22-36μm) and B- granules (2-3μm) (Perez and Bertoft 2010). Maltogenic amylase -A action on NWS showed an entirely different erosion pattern on A- and B- granules. B-granules showed few pinholes while A- granules were extensively damaged with cavities formation at the end of 24h pointing to greater resistance offered by the outer layers and a more susceptible inner region (Figure 2.13). Action of maltogenic amylase -B on NWS started with surface erosion on B-granules and formation of cavities with a hollow structure during later stages while A-granules did not show any significant damage (Figure 2.14) indicating the structural differences between both types of granules and their relative susceptibility to enzyme action. Deep cavities formation in large A-granules indicates greater susceptibility of internal structures to enzyme action. Both enzymes were able to degrade A- and B-granules simultaneously irrespective of the particle size of granules. This is in contrast with previous studies (Franco and Ciacco 1992, 1998; Knutson et al. 1982; Manelius et al. 1997; Tang et al. 2004) where small granules were found to be more susceptible to enzyme action than large granules. α-amylase action on wheat starch granules resulted in greater damage to large A- granules. Action of α-amylase on wheat starch granules

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resulted in holes formation along with equatorial groove like structure and multiple pitting along surface (Blazek and Gilbert 2010). Large A- granules are flat and possess an equatorial groove while small B- granules are rounded (Jane et al. 1994; Manelius et al. 1997; Morrison and Gadan 1987). In a study (Gallant et al. 1972) pancreatic amylase action on wheat starch granules was investigated. Enzyme action resulted in endocorrosion through holes at the groove, exocorrsion and radial degradation between characteristic growth rings. Action of *Bacillus subtilis* α-amylase resulted mainly in endocorrsion of large granules through holes present on the equatorial groove while keeping most of the granules intact. Also the enzyme caused selective hydrolysis of granules with composition of granules remaining unchanged and thus possessing granule by granule attack (Colonna et al. 1988). Large granules have increased amylose deposits in the outer parts perpendicular to the equatorial groove (Morrison and Gadan 1987). Sarikaya et al. (2000) concluded that the degradation pattern of limited hydrolysed native starches by α-amylase is starch specific. Exo acting β-amylase was found to be less effective in hydrolyzing native starch granules than α-amylase. Pores found on the surface of granules of corn, sorghum and millet starches and along the equatorial groove of large granules of wheat, rye and barley starches serve as openings for enzyme to access inner parts of granules (Fannon et al. 1992). SEM images of α-amylase hydrolysed barley starches showed starch degradation preferably at the equatorial groove while greater numbers of large holes were observed on starches hydrolysed by α-glucosidase and α-amylase combination (Sun and Henson 1990). A granule by granule attack on wheat starch granules where an attacked granule was completely hydrolyzed by α-amylase, has been reported in the past (Colonna et al. 1988).

Thermal properties of granular starch hydrolysates

Differential scanning calorimetry has been used to estimate the degree of order in starch hydrolyzates. Gelatinization enthalpy (ΔH gel) a measure of crystalline disorder and disruption of double helices, partial solubilization of amylose and glucan chain-water complexes and chain chain interactions (Bogracheva et al. 2002). Gelatinization properties of granular starches and products produced by the enzyme are shown in Tables 2.9 to 2.10.

Both enzymes resulted in reduced gelatinization enthalpies and increased gelatinization temperatures of granular starches. Maltogenic amylase -A reduced ΔH of WMS by 12.9% and 51.0% compared to control; ΔH of NWS by 22.5% and 62.5% compared to control at the end of

4h and 24h, respectively. Maltogenic amylase -B reduced ΔH of WMS by 20% and 62.7% compared to control; ΔH of NWS by 20.5% and 58.8% compared to control at the end of 4h and 24, respectively. At the end of 24h, narrowing down of the gelatinization peak was observed for both starches and enzymes which indicate the decrease in crystalline heterogeneity or an increase in homogeneity in crystallites size and stability (Biliaderis 1992). Different starches exhibit different gelatinization temperatures depending on their molecular structure, starch composition and granular architecture (Rezzoug et al. 2011). Waxy starches shows a highly symmetric gelatinization peak due to the melting of pure A type crystallites (Blazek and Gilbert. 2010) while wheat starch shows a symmetric peak with second half of the peak showing a prolonged decay and second enthalpic transition owing to dissociation of amylose-lipid complexes and melting of V type crystallites (Matveev et al. 2001).

Increased gelatinization temperature as seen in our study indicates that the crystallites became more resistant to melting as a result of enzyme action because granules with more stable crystallite melt at higher temperature (Evans and Haisman 1982). The increase in onset and gelatinization temperatures as result of starch hydrolysis was attributed to hydrolysis of branching points and unwinding of double helices due to which crystalline regions become less connected or restrained by amorphous regions (Blazek and Gilbert 2010). Increase in thermal transition temperatures of lentil starches which have C type crystallinity due to α-amylolysis was attributed to decoupling of crystallites from the amorphous regions as a result of hydrolysis of the bulk and intercrystalline amorphous regions therefore, requiring a higher temperature for crystallite melting. However, unchanged enthalpy was attributed to the resistance of double helices in the amorphous and crystalline domains toward amylase action (Hoover 1995). A positive correlation between higher proportion of shortest A chains (DP 5-10) with imperfect crystalline structure was reported because they cannot form stable double helices and are more likely to be attacked by enzymes (Gidley and Gidley. 1987; Zhang et al. 2006).

A higher ΔH gel of native starches is due to greater degree of ordered structure and thus a lower availability of α-glucan chains to enzyme hydrolysis. A decrease in ΔH gel of starches upon enzyme digestion, observed in our study, indicates a decrease in ordered structure and hence starch chains availability to enzyme action. Also ΔH of amylose lipid complexes of hydrolyzed NWS of both enzymes indicates these complexes were formed as well as degraded during the course of hydrolysis. This means the amylose fragments produced upon enzyme

digestion were further forming complex with lipid already present in the starch and were also simultaneously being degraded.

X-ray diffraction of granular starch hydrolysates

X-Ray diffraction was used to determine the changes in crystallinity after enzyme hydrolysis. Both WMS and NWS exhibited typical A-type pattern (Figures 2.15 to 2.16). NWS, in addition, showed an amylose-lipid complex peak. With an increase in hydrolysis time, a decrease in crystallinity was observed for both starches hydrolysed by both enzymes. This indicates that enzymes were able to hydrolyze both amorphous and crystalline regions simultaneously which became apparent at the end of 24h where no crystalline peaks were observed. NWS hydrolyzates showed a greater loss of crystallinity than WMS hydrolysates at the end of 24h with almost complete disappearance of peaks. Contrary to lower DH (%) of WMS and NWS achieved by both enzymes, degree of crystallinity decreased to a greater extent. According to (Colonna et al. 1992), crystallinity and accessibility are main factors limiting native starch hydrolysis. Our results showed that maltogenic amylases were able to act on these crystalline regions and thereby decreasing crystallinity. For granular normal maize starch (5%) hydrolysed by bacterial α-amylase from *Anoxybacillus flavothermus* although a high DH (%) of about 82 was achieved, crystallinity decreased to a lesser extent (Tawil et al. 2012). Also, amylose lipid peak at 2θ around 20$^{°}$ was found to be unstable suggesting that both enzymes were able to hydrolyze these amylose-lipid complexes. This is quite interesting since amylose-lipid complexes are resistant to α-amylase action and remains stable (Gernat et al. 1993; Kwasniewska et al. 2008; Lauro et al. 1999). Tawil et al. (2012) concluded that amylose lipid complexes are more resistant to enzyme action at low starch concentration (5%) while crystalline structures become more susceptible to enzyme action at high starch concentration (31%). However, in our study we found that maltogenic amylases can hydrolyze amylose lipid complexes even at lower starch concentration (2%). Similarly PPA can hydrolyse mutant maize starches with A – and Vh-types (Gerard et al. 2001). In vitro digestion of these complexes by α-amylase when used in excess amounts or longer hydrolysis time has also been reported (Biliaderis and Galloway 1989; Faisant et al. 1993; Holm et al. 1983). A side by side digestion pattern of amorphous and crystalline regions of normal maize starch have been proposed where both regions were digested at the same rate (Zhang et al. 2006). Rate of starch digestion is influenced by degree of starch

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crystallinity, crystalline type and amylase is reported to preferably attack non crystalline regions in starch (Planchot et al. 1997). α-amylase from PPA hydrolysed both amorphous and crystalline parts at the same rate as shown by insignificant increase in crystallinity after hydrolysis (Gerard et al. 2001). Digestion of both crystalline and amorphous regions of wheat starch (Colonna et al. 1988) and maize mutant starches (Gerard et al. 2001) by amylase was observed while α-amylase from *Anoxybacillus flavothermus* (Tawil et al. 2012) and fungal α-amylase from *Rhizomucor* sp. (Tawil et al. 2011) was found to be digesting crystalline part of maize starch efficiently. Endo enzyme, glucoamylase acts differently by increasing crystalline character of hydrolysed waxy maize starches (Aggarwal and Dollimore 1998; Brewer et al. 2012).

In consistent with the MW distributions of hydrolysed granular starches at 24h, where AP fraction was degraded to a greater extent, and an almost complete disappearance of crystalline peaks was observed. This is because AP is largely responsible for supporting the crystalline domains in starch granules (Perez and Bertoft 2010) and its degradation upon enzyme digestion led to decrease in crystallinity.

Conclusions

From the hydrolysis patterns of both enzymes, it was observed that different oligosaccharides compositions and MS distributions were produced for granular and swollen starches. Irrespective of starch type, starch form, swelling temperature and hydrolysis time, maltogenic amylase -B action caused a greater DH than maltogenic amylase -A due to its higher endo activity. WMS was hydrolyzed more than NWS however for the native granular starches, DH (%) of NWS was found to be higher than that of WMS at the end of 4h and 24h for maltogenic amylase -A and 24h for maltogenic amylase -B. Maltogenic amylase -B displayed a higher DMA on both granular and swollen starches than maltogenic amylase -A. Interestingly, their LICA was found to be independent of substrate accessibility as seen through similar MS distributions profiles of hydrolyzed swollen starches. For granular starches, both enzymes followed a heterogenous and localized attack with damage to few granules with most granules still largely intact. Stable crystallites were formed upon enzyme digestion of starch granules as seen through DSC results where a decrease in gelatinization enthalpy and increased gelatinization temperature was observed. Both enzymes were able to hydrolyze amorphous and crystalline regions in starch granules as shown by decrease in crystallinity by XRD. These results

indicate that LICA of both enzymes would be independent while DMA would increase with increase in degree of starch swelling during baking. Also heterogeneous attacks on starch granules by both enzymes where few granules are attacked keeping mostly intact means the overall starch composition will largely remain the same suggesting that starch structure would be strong enough not to affect bread resiliency and volume even after enzyme has acted upon starch during baking process.

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Figure 3.1   Molecular size distributions of the native granular starches hydrolyzed by maltogenic amylase –A (a) waxy maize starch; (b) normal wheat starch

(a)

(b)
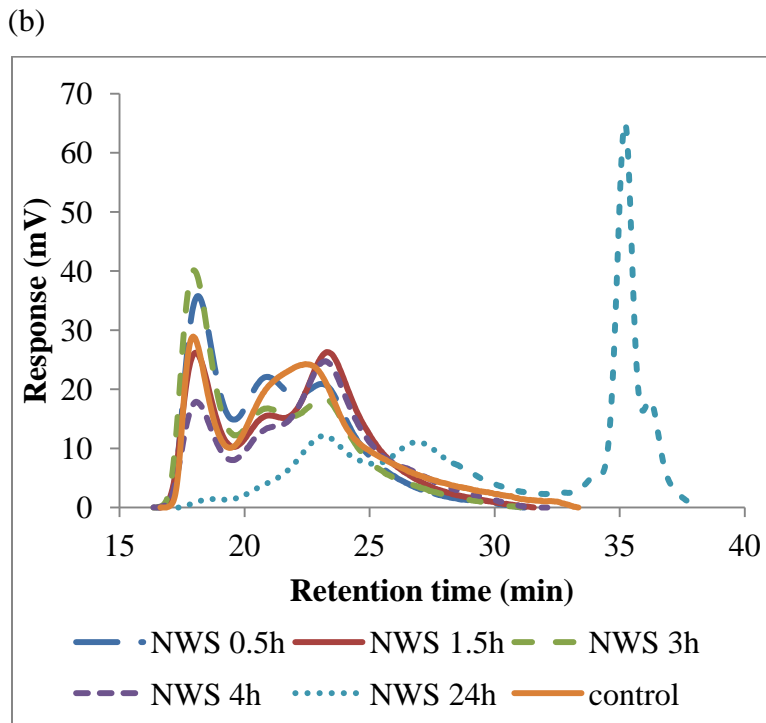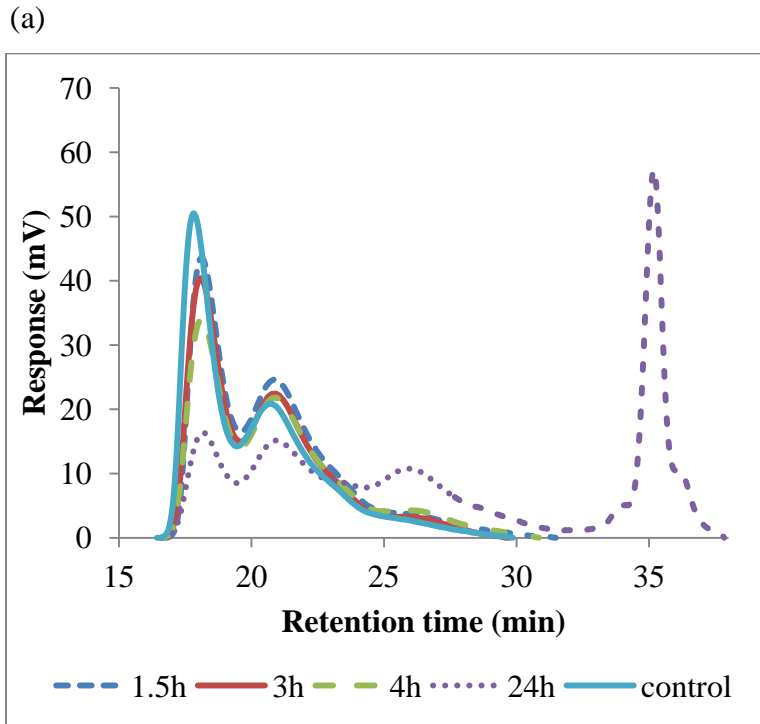
Fig. 3.2 Molecular size distributions of the native granular starches hydrolyzed by maltogenic amylase -B (a) waxy maize starch; (b) normal wheat starch

(a)
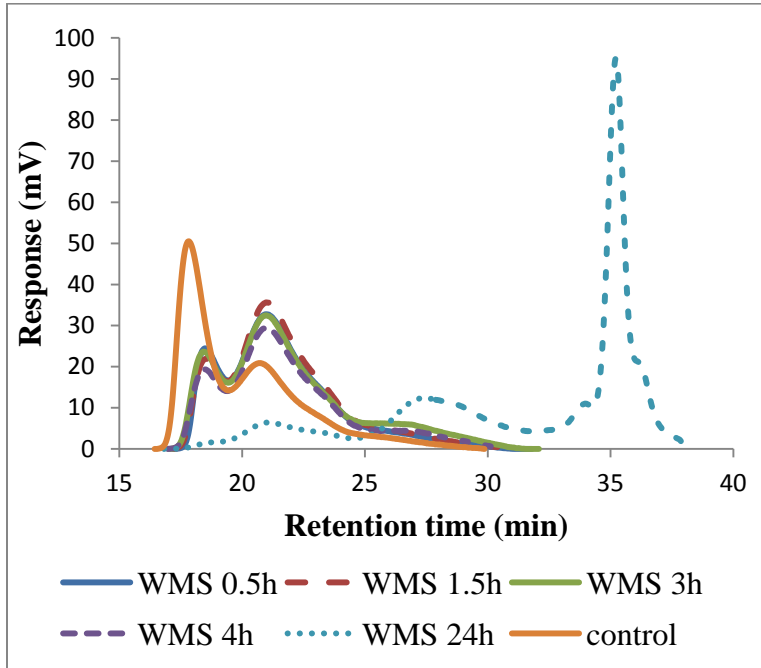
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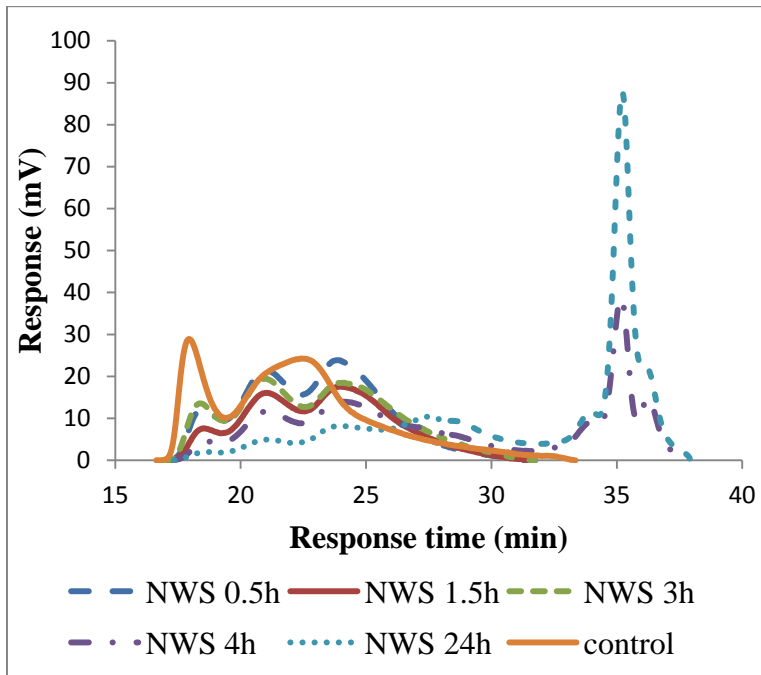
Fig. 3.3 Molecular size distributions of starches swelled at 70°C, cooled to 62°C and hydrolyzed by maltogenic amylase -A (a) waxy maize starch; (b) normal wheat starch

(a)
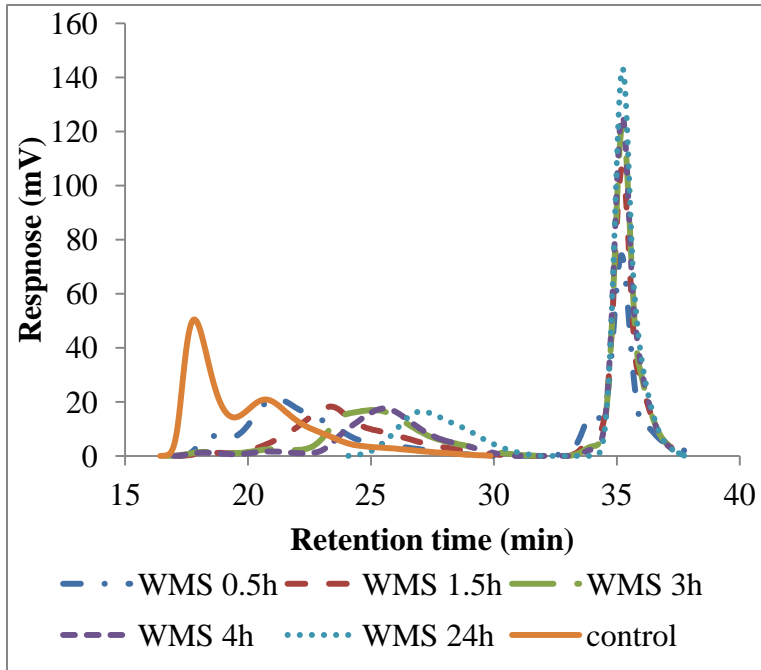
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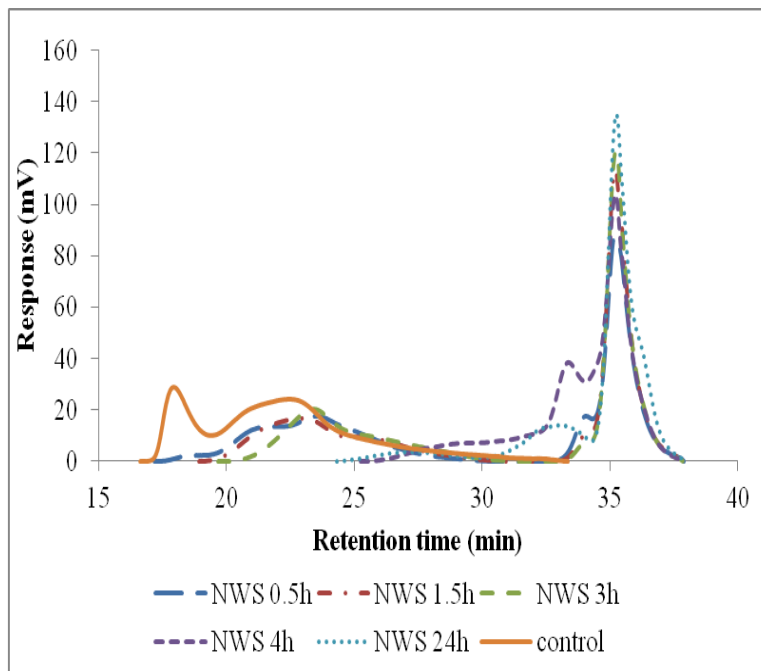
Figure 3.4. Molecular size distributions of starches swelled at 70°C, cooled to 55°C and hydrolyzed by maltogenic amylase –B (a) waxy maize starch; (b) normal wheat starch

(a)
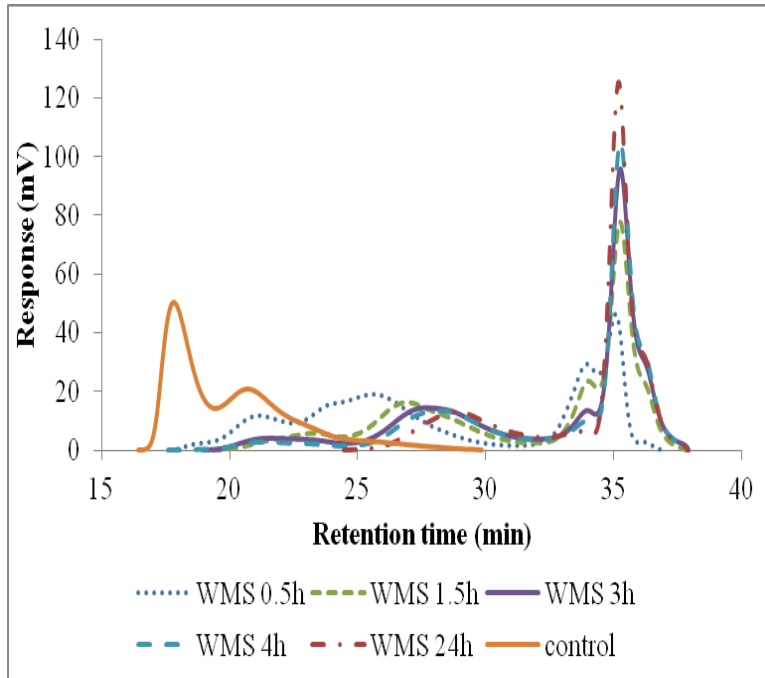
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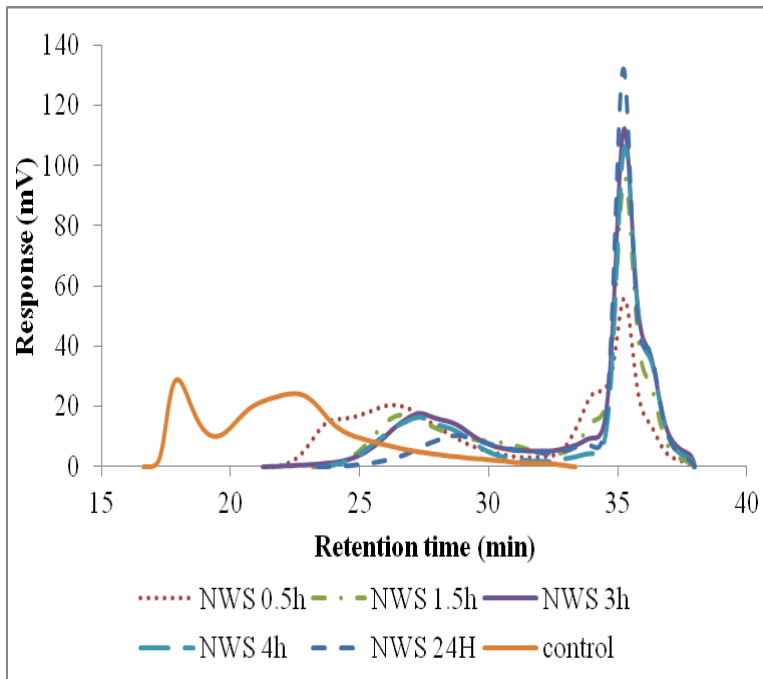
Figure 3.5 Molecular size distributions of starches swelled at 80°C, cooled to 62°C and hydrolyzed by maltogenic amylase -A (a) waxy maize starch; (b) normal wheat starch

(a)
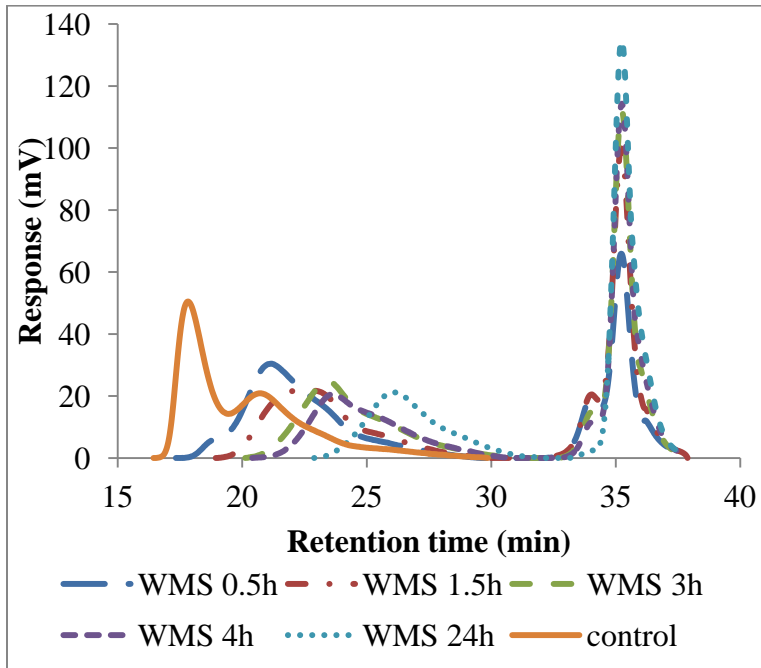
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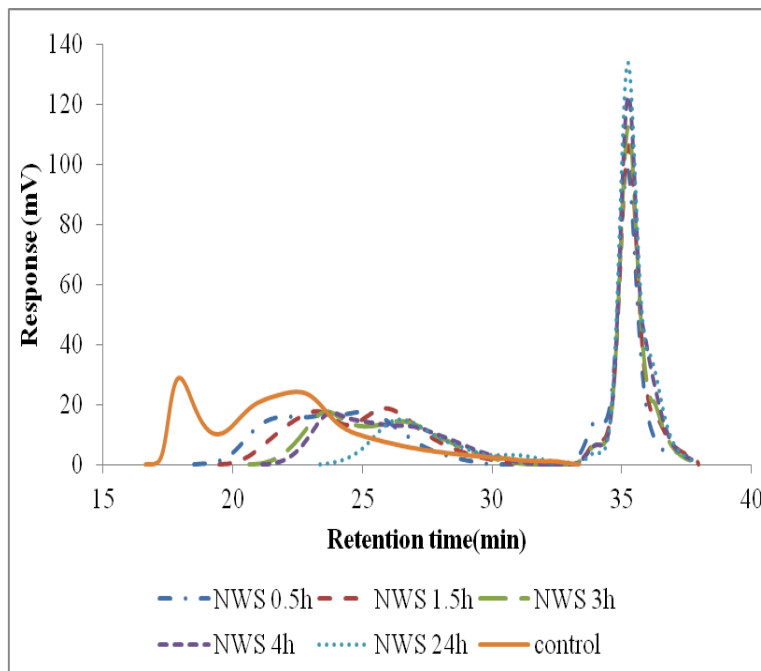
Figure 3.6 Molecular size distributions of starches swelled at 80°C, cooled to 55°C and hydrolyzed by maltogenic amylase -B (a) waxy maize starch; (b) normal wheat starch

(a)
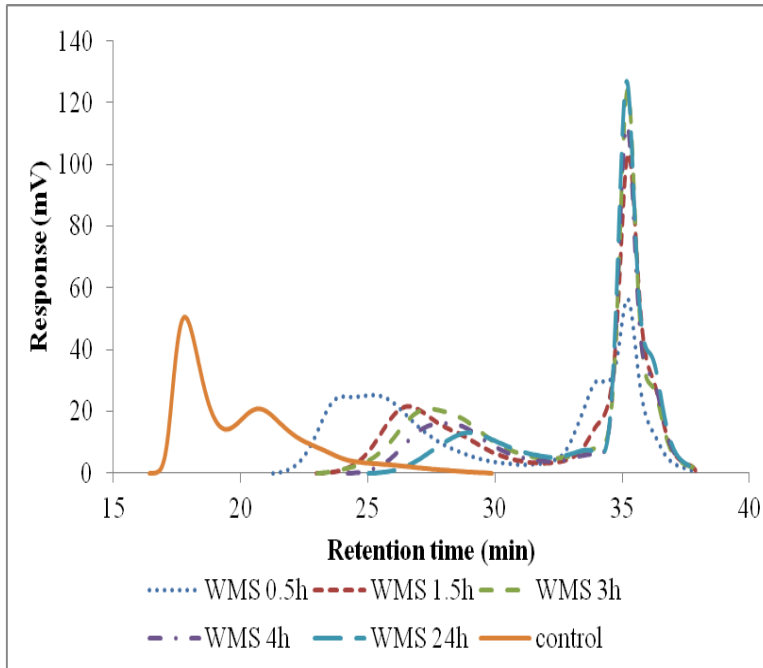
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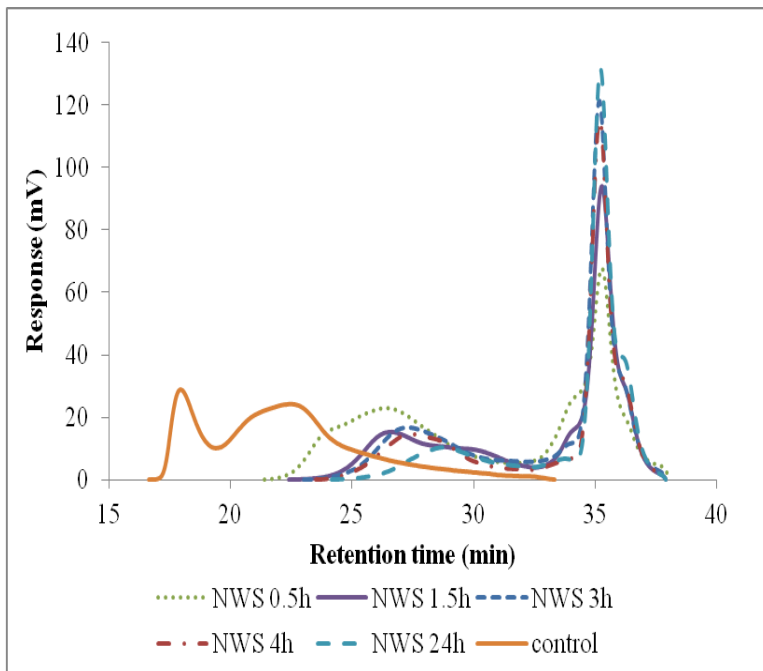
Figure 3.7 Molecular size distributions of starches swelled at 90°C, cooled to 62°C and hydrolyzed by maltogenic amylase -A (a) waxy maize starch; (b) normal wheat starch

(a)
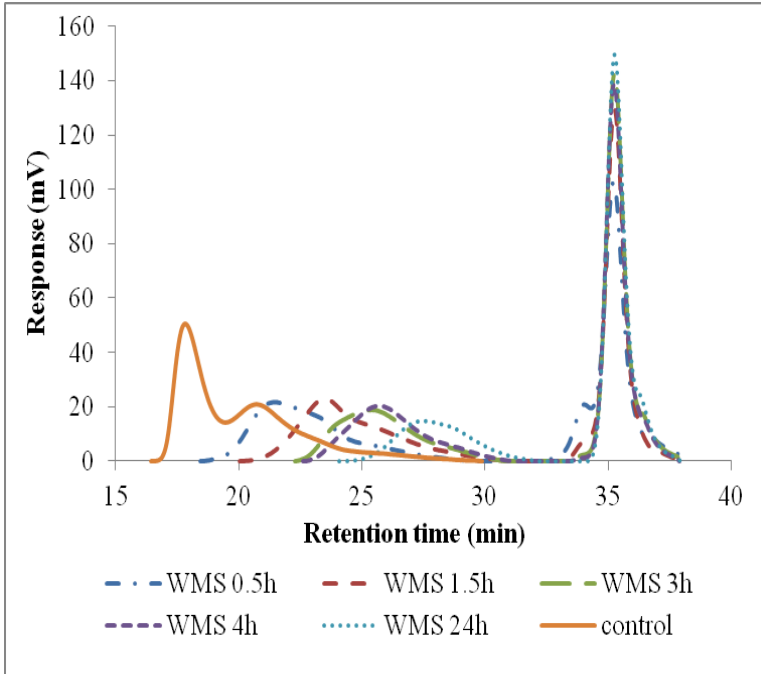
(b)
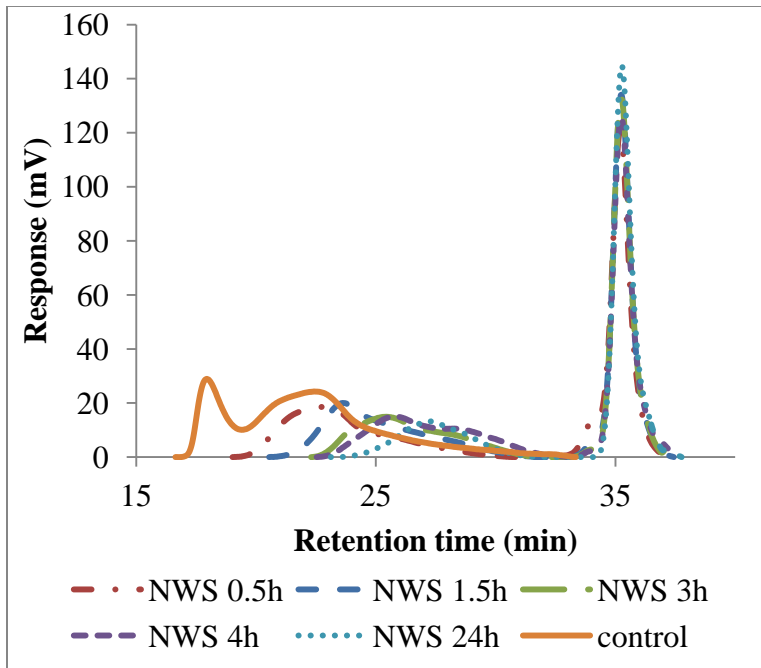
Figure 3.8 Molecular size distributions of starches swelled at 90°C, cooled to 55°C and hydrolyzed by maltogenic amylase-B (a) waxy maize starch; (b) normal wheat starch

(a)
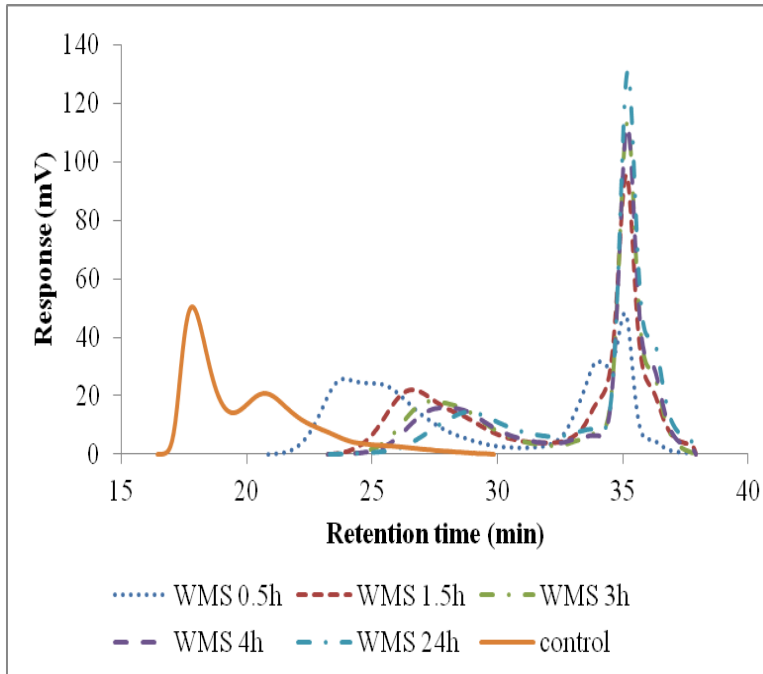
(b)
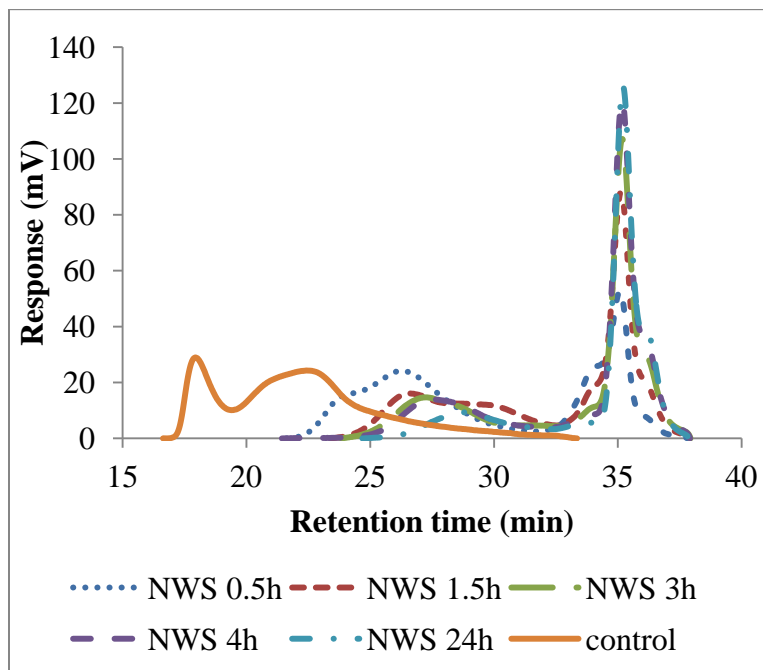
Figure 3.9 Microscopic images of the native granular starches hydrolyzed by maltogenic amylase-A (a) waxy maize starch (control, 0.5h, 4h, 24h) ; (b) normal wheat starch (control, 0.5h, 4h, 24h)

(a)
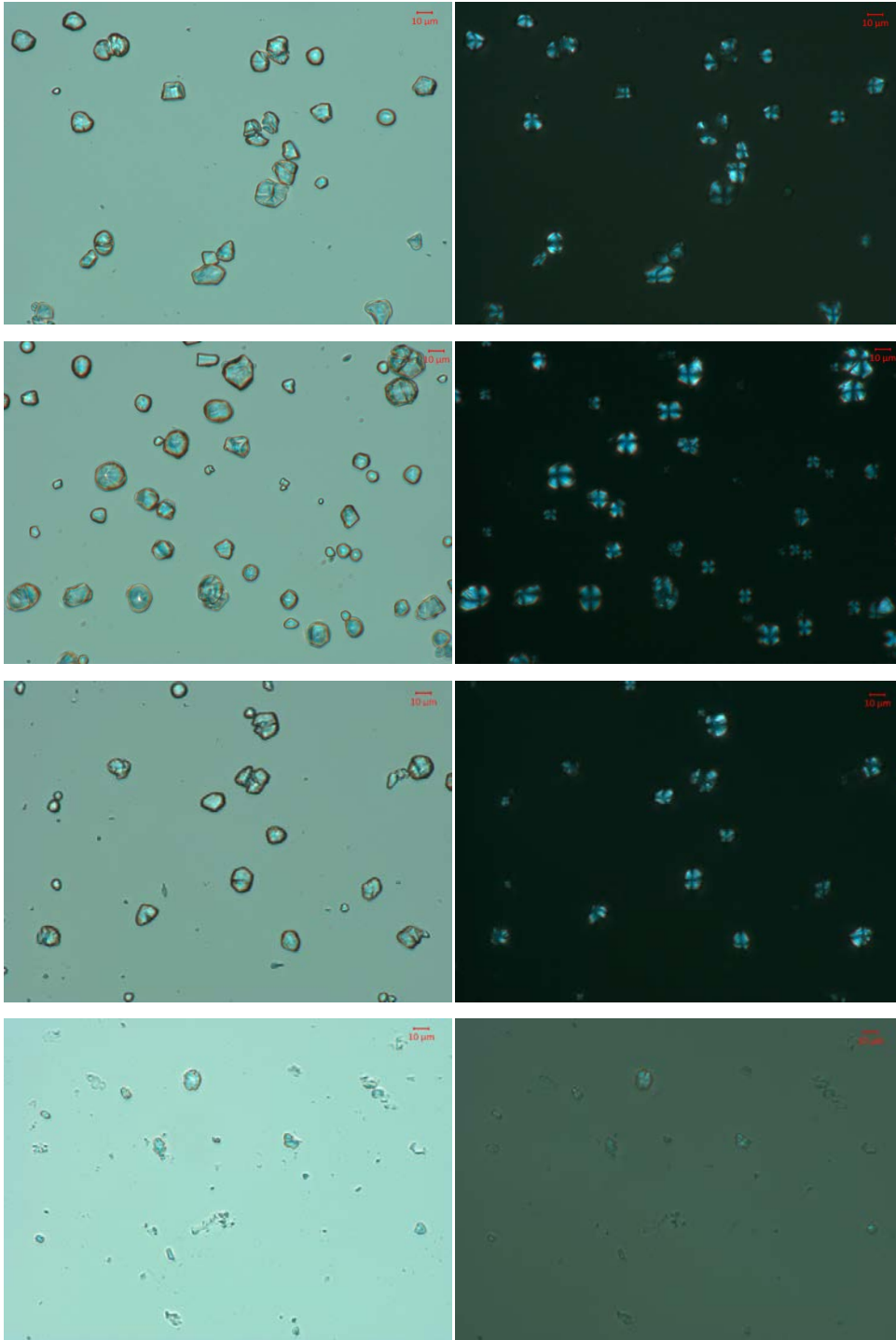
(b)
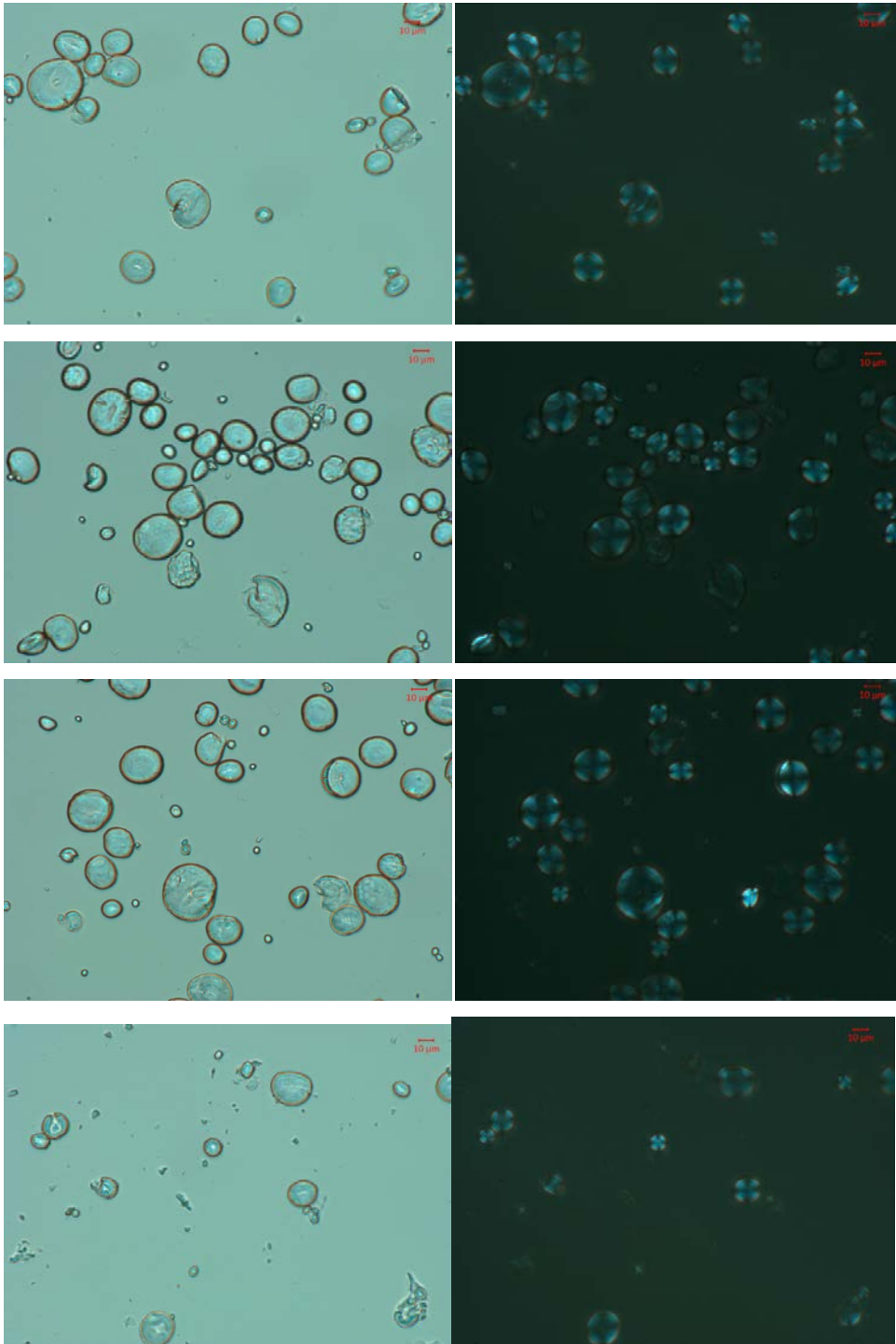
Figure 3.10 Microscopic images of the native granular starches hydrolyzed by maltogenic amylase- B (a) waxy maize starch (control, 0.5h, 4h, 24h) ; (b) normal wheat starch (control, 0.5h, 4h, 24h)

(a)
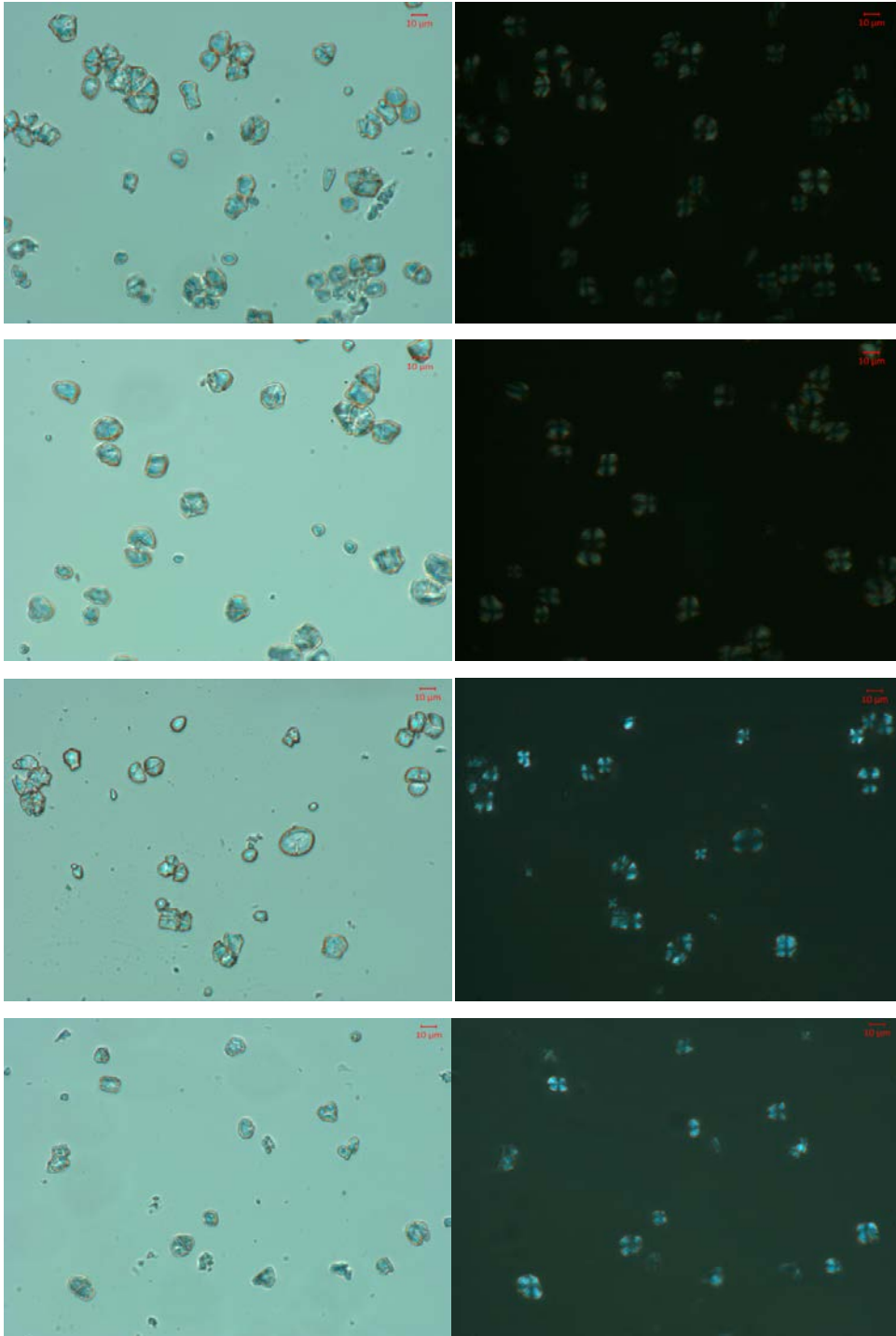
(b)
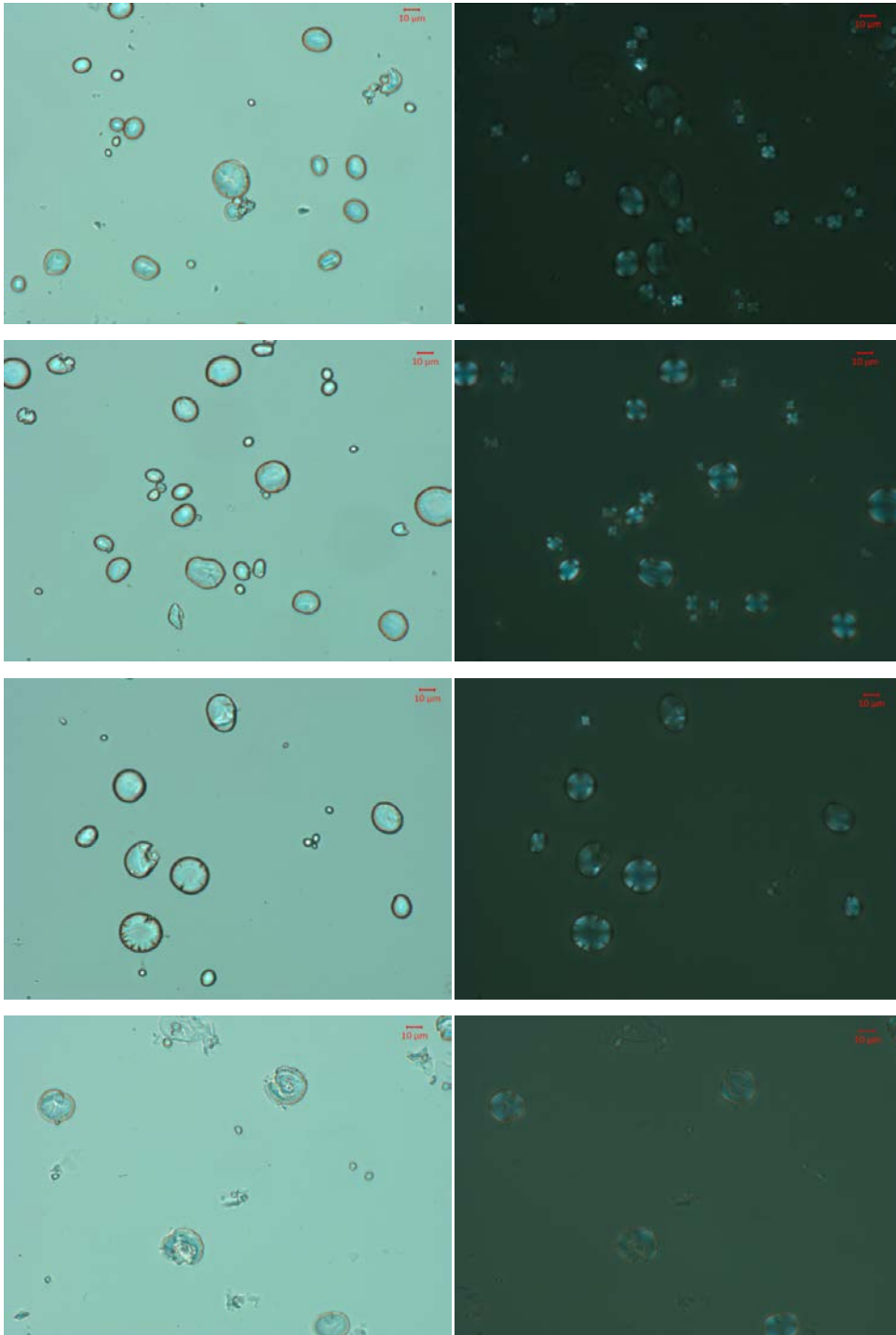
Figure 3.11 Microscopic images of starches swelled at 70°C, cooled to 62°C and hydrolyzed by maltogenic amylase -A (a) waxy maize starch (control, 0.5h, 4h, 24h); (b) normal wheat starch(control, 0.5h, 4h, 24h)

(a)
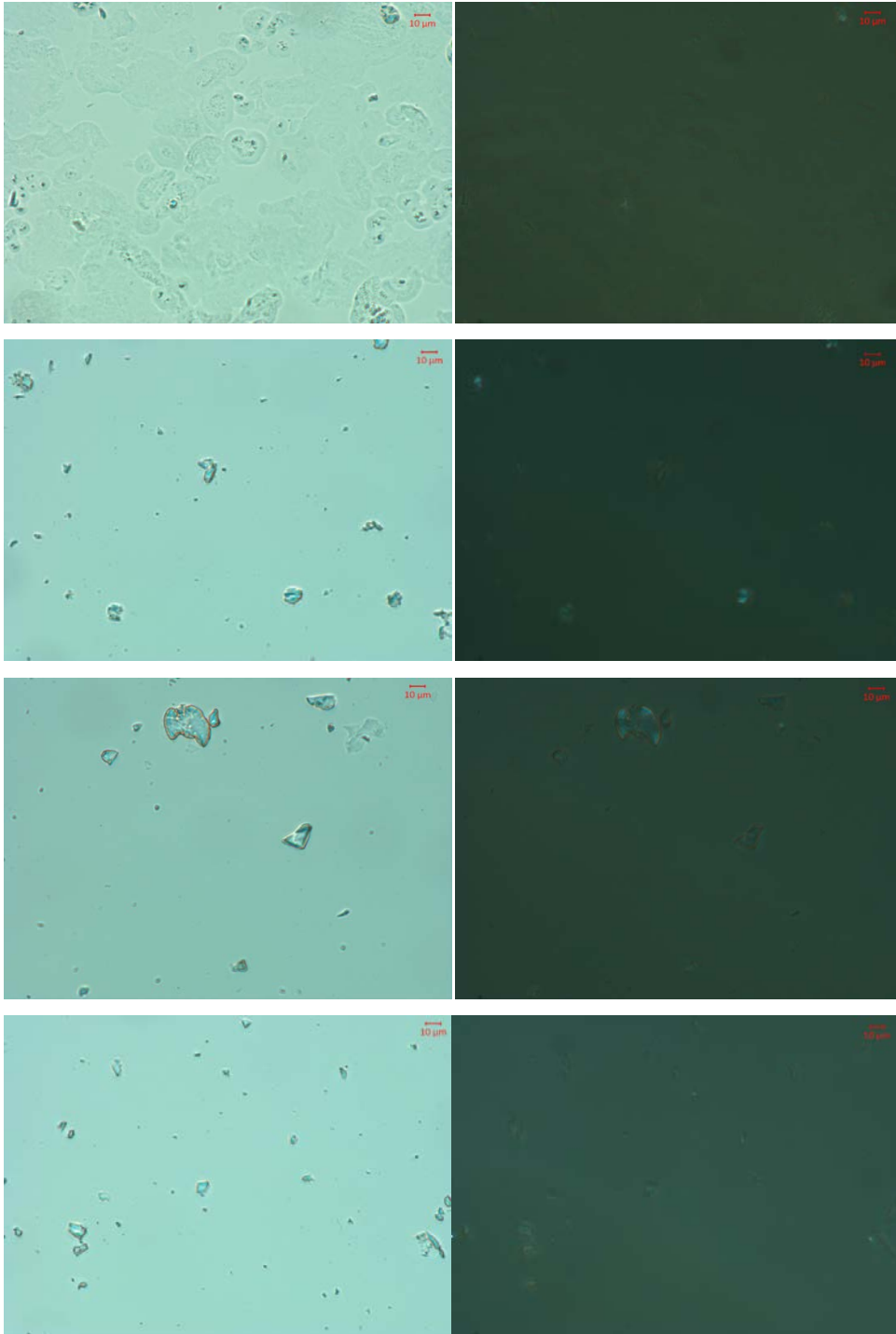
(b)
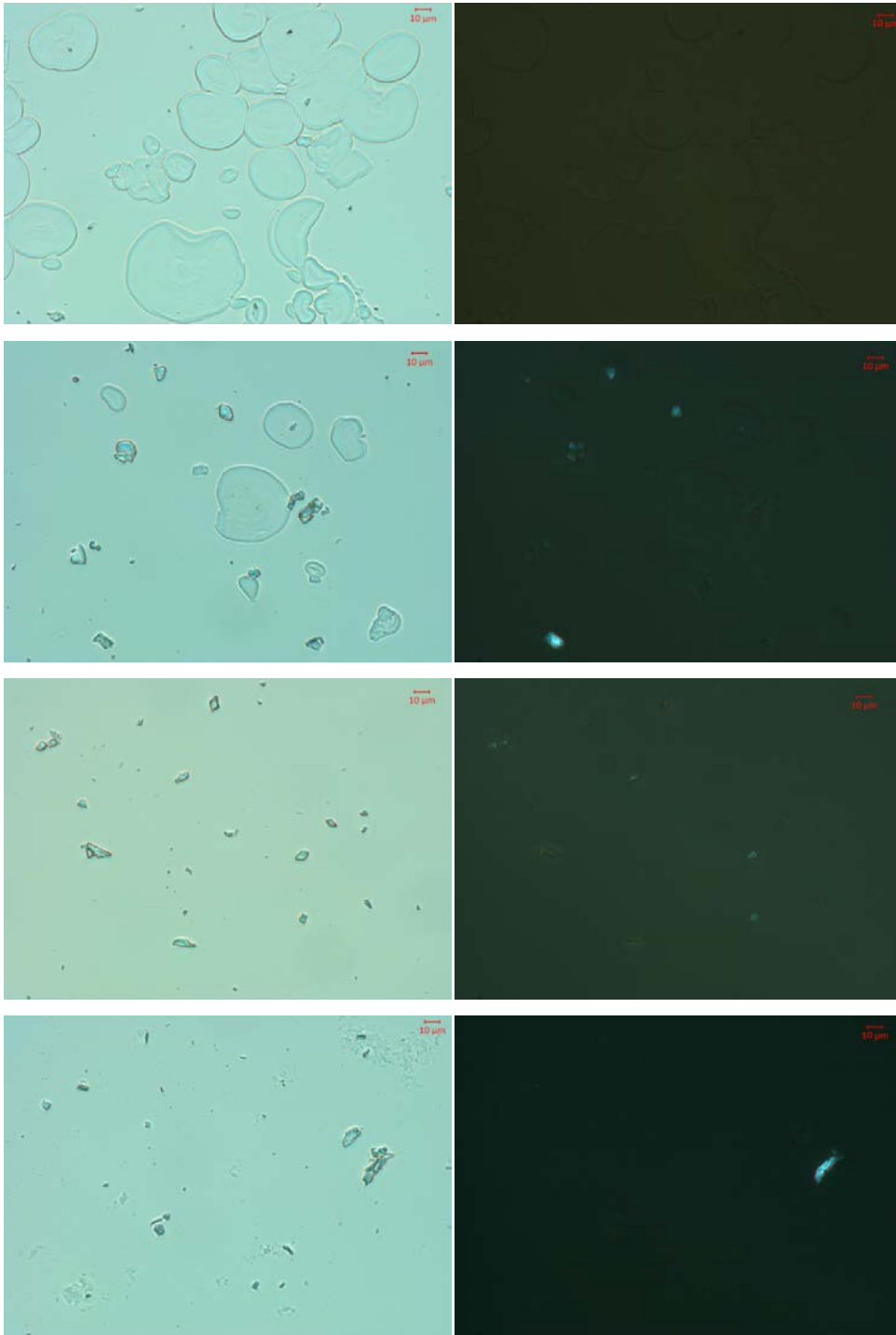
Figure 3.12 Microscopic images of starches swelled at 70°C, cooled to 55°C and hydrolyzed by maltogenic amylase -B (a) waxy maize starch (control, 0.5h, 4h, 24h); (b) normal wheat starch(control, 0.5h, 4h, 24h)

(a)
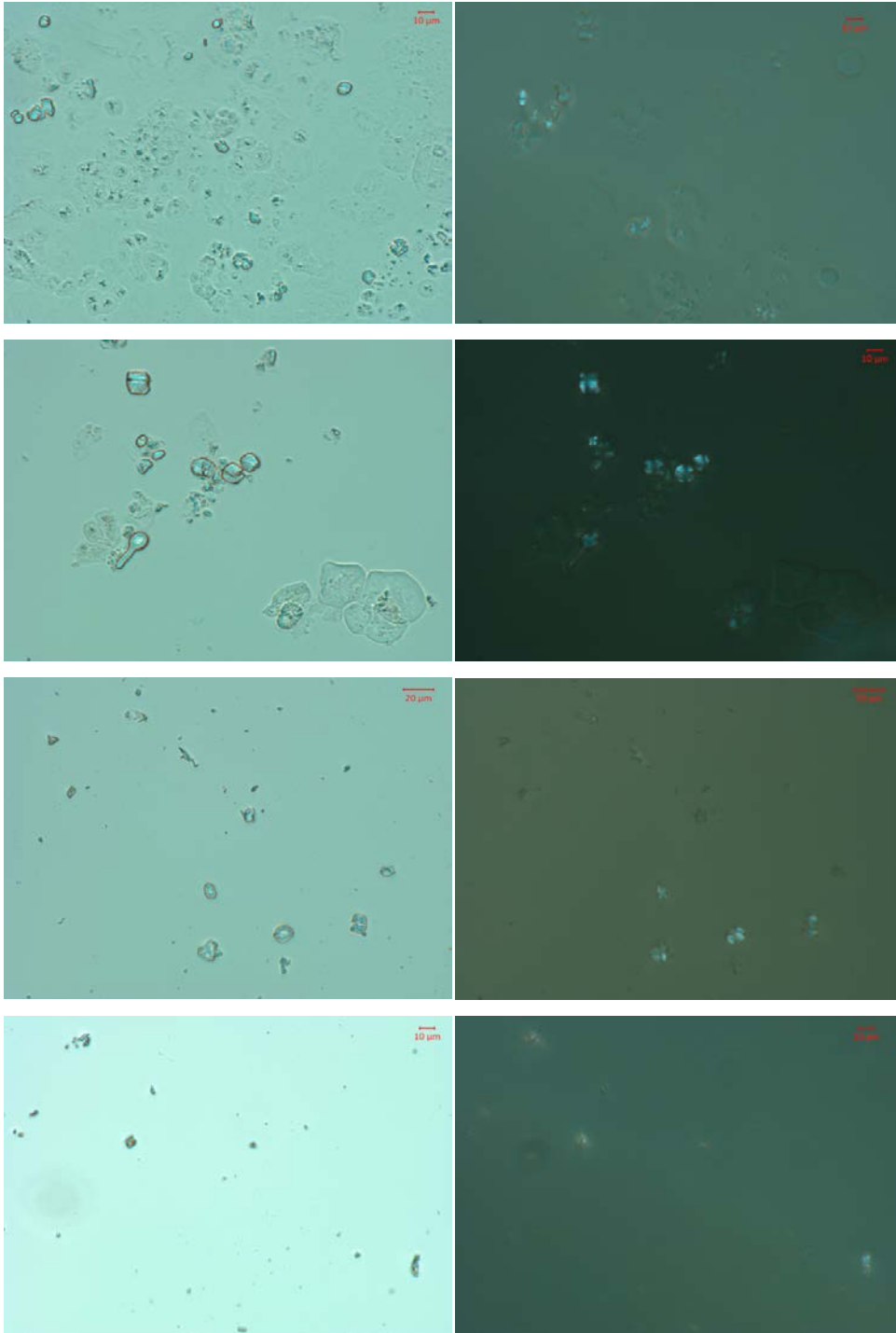
(b)
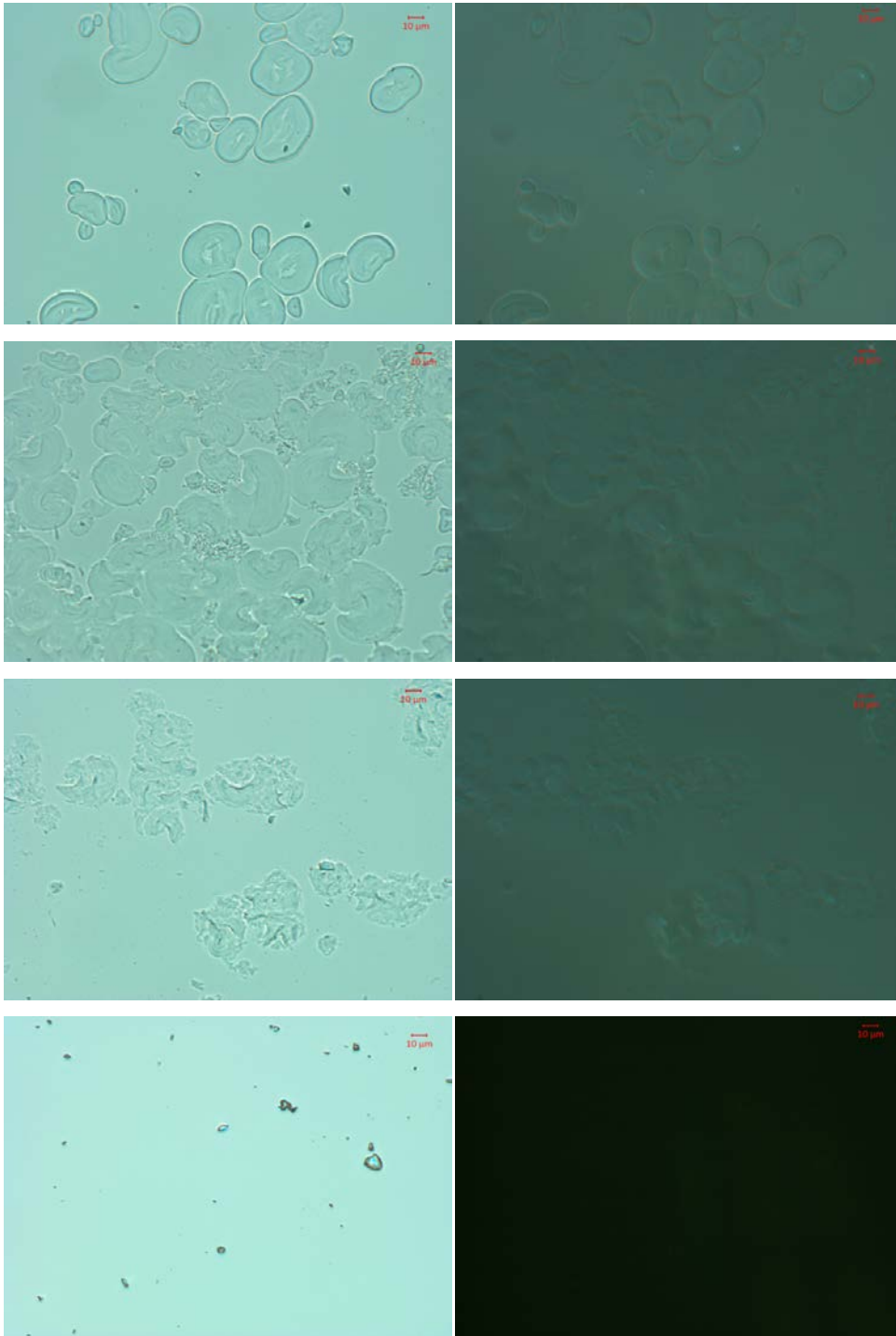
Figure 3.13 Scanning electron micrographs of native granular starches hydrolyzed by maltogenic amylase -A (a) waxy maize starch (control, 0.5h, 4h and 24h) (b) normal wheat starch (control, 0.5h, 4h and 24h)

(a)
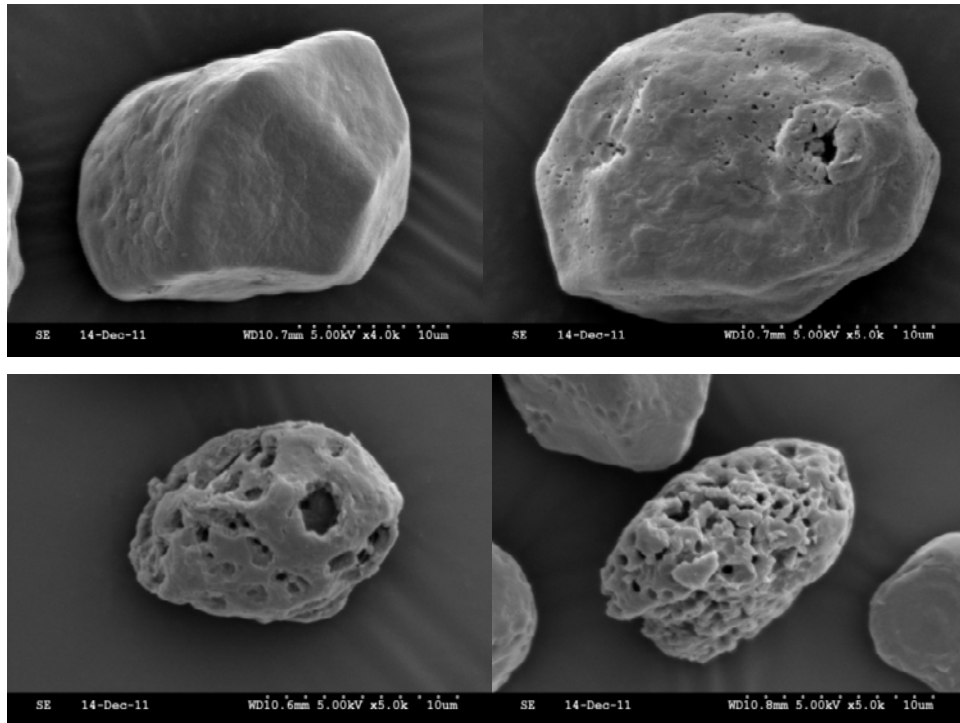
(b)
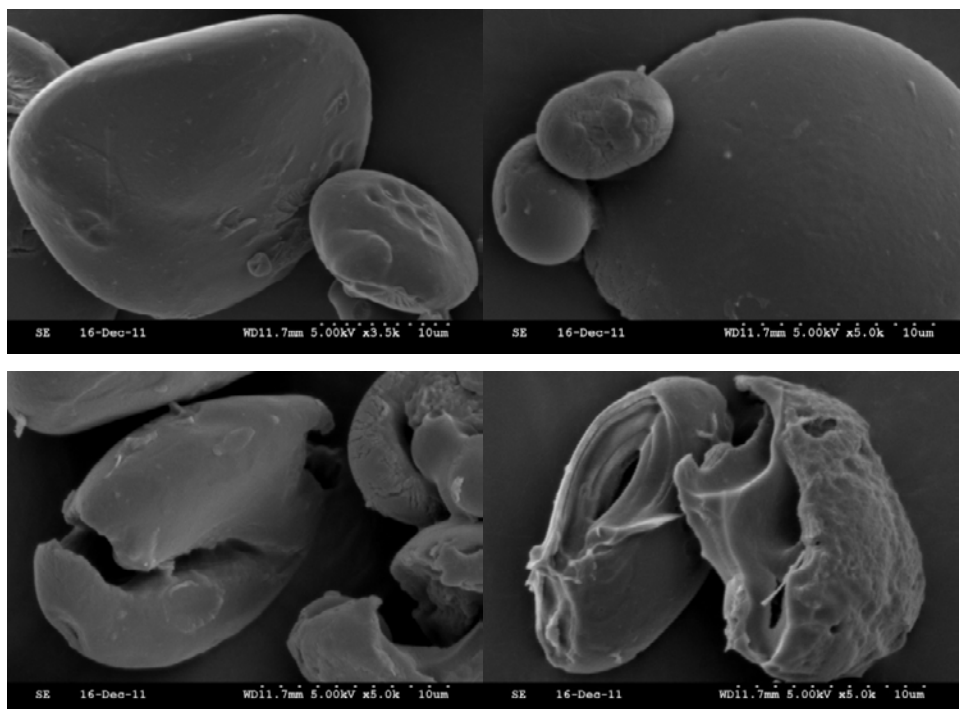
Figure 3.14 Scanning electron micrographs of native granular starch hydrolyzed by maltogenic amylase -B (a) waxy maize starch (control, 0.5h, 4h and 24h) (b) normal wheat starch (control, 0.5h, 4h and 24h)

(a)
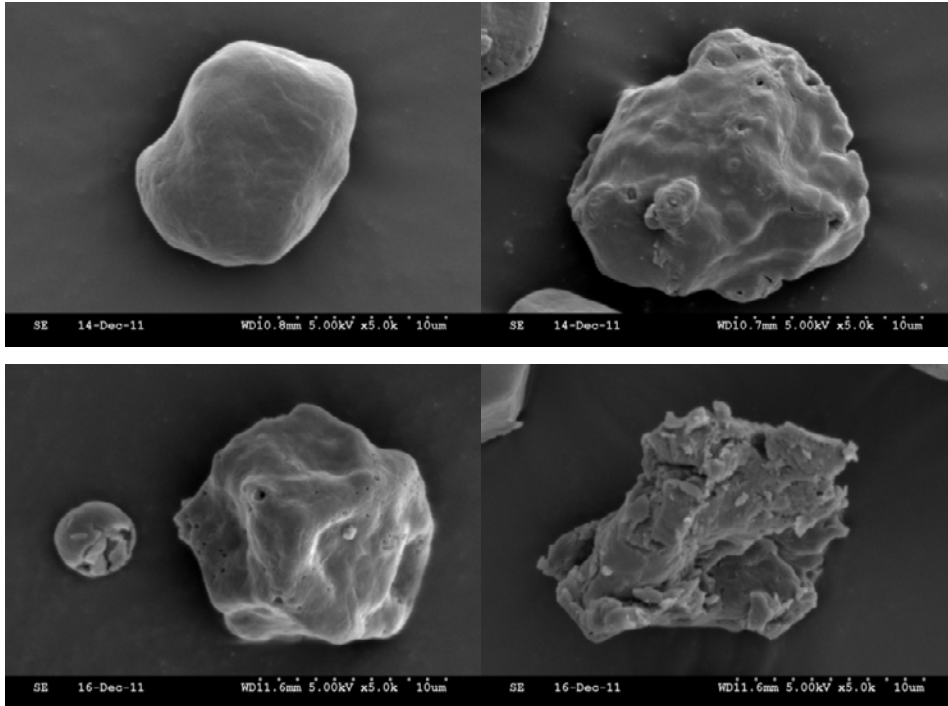
(b)
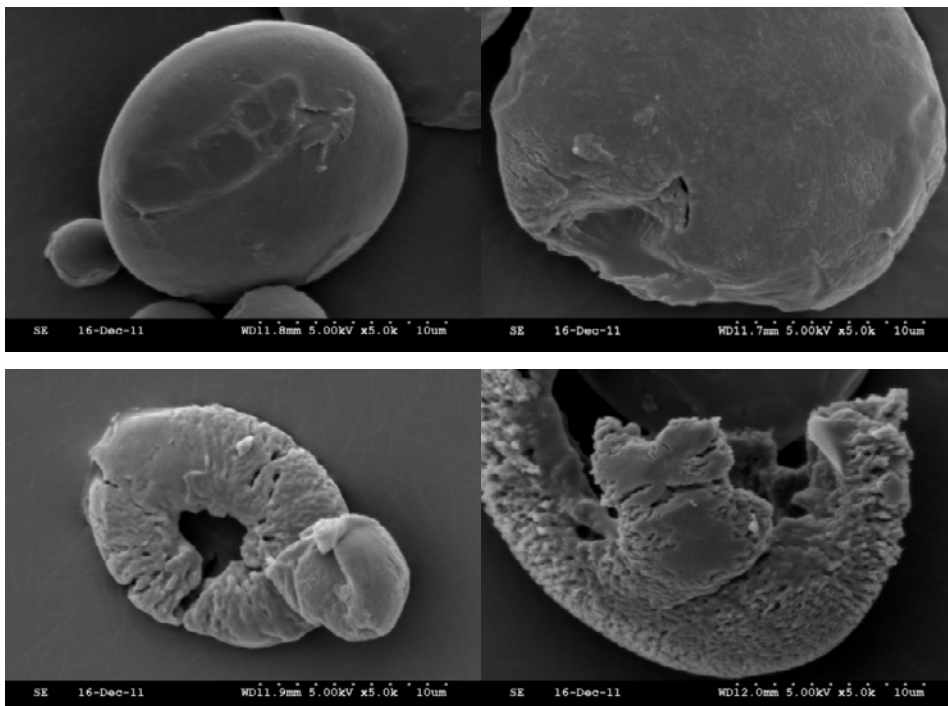
Figure 3.15 X-ray diffraction patterns of native granular starches hydrolyzed by maltogenic amylase -A (a) waxy maize starch (b) normal wheat starch.

(a)
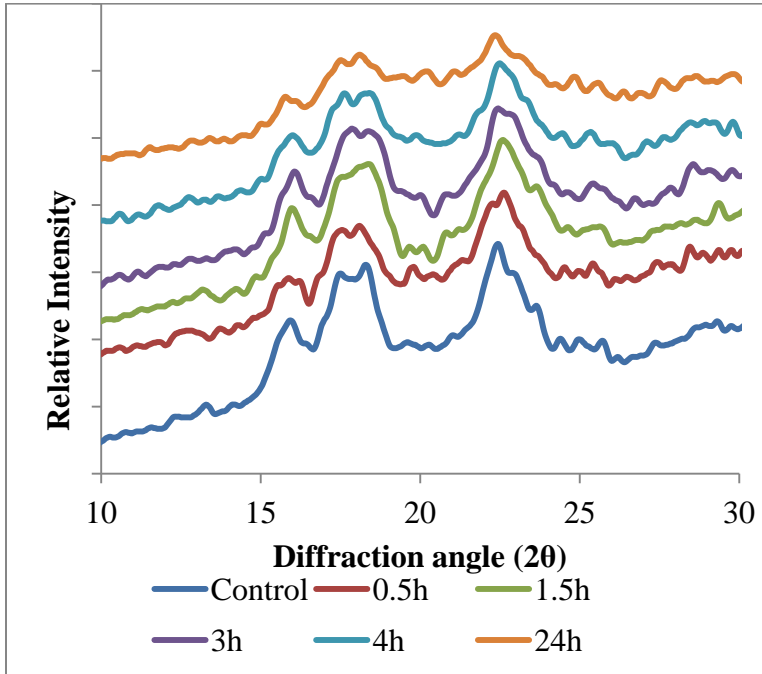
(b)
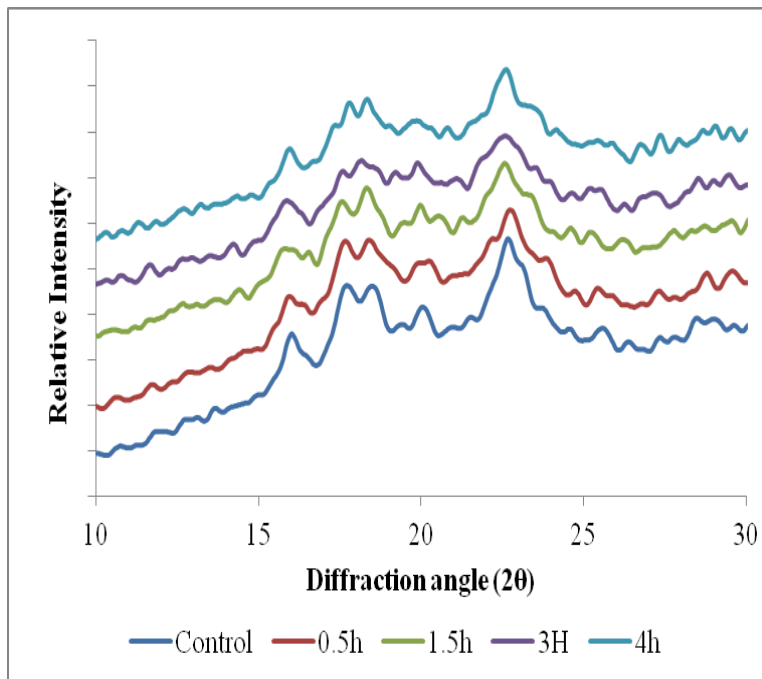
Figure 3.16 X-ray diffraction patterns of native granular starches hydrolyzed by maltogenic amylase -B (a) waxy maize starch (b) normal wheat starch.

(a)
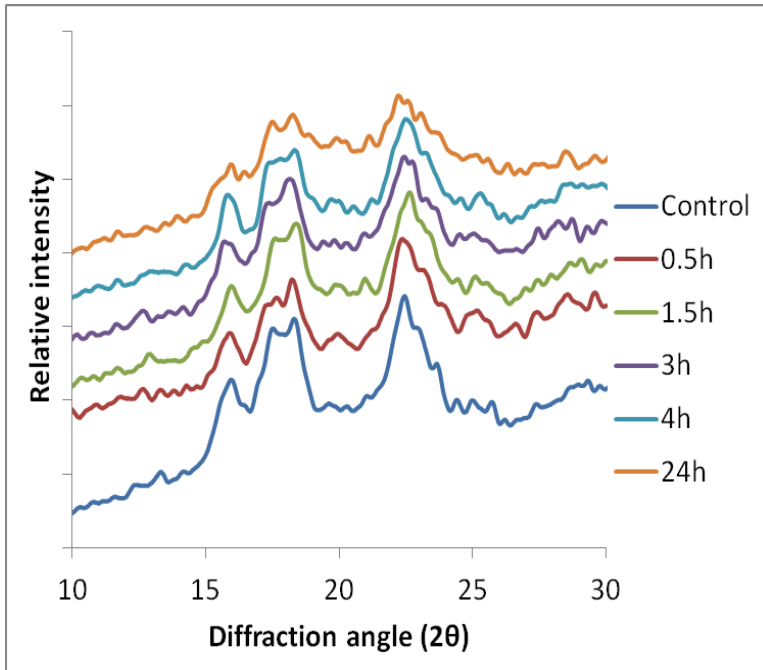
(b)
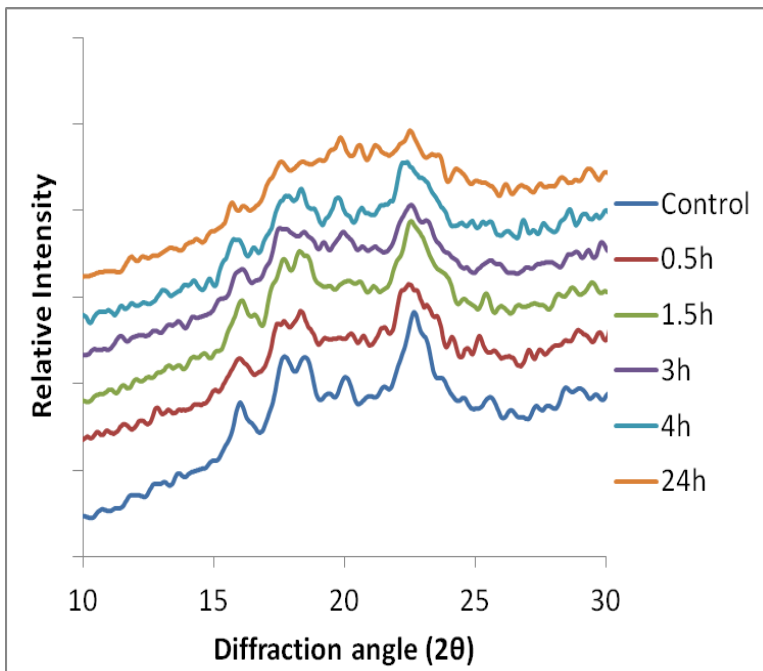
Table 3.1 Percentage sugars (based on initial starch weight) in granular starches hydrolyzed by maltogenic amylase -A *

Hydrolysis time (h)	Waxy maize starch hydrolysates					DH (%) **
	DP- 1	DP- 2	DP- 3	DP- 4	DP- 5	
0.5	0.0	1.3	0.0	0.3	0.0	1.7 ± 0.2^{ef}
1.5	0.1	2.4	0.0	0.4	0.1	3.0 ± 0.1^{e}
3	0.2	3.8	0.2	0.5	0.1	$4.7 \pm 1.^{d}$
4	0.2	4.7	0.1	0.5	0.1	5.7 ± 0.3^{de}
24	0.3	9.9	0.1	0.9	0.1	11.3 ± 0.9^{b}

Hydrolysis time (h)	Normal wheat starch hydrolysates					DH (%) **
	DP- 1	DP- 2	DP- 3	DP- 4	DP- 5	
0.5	0.2	0.9	0.0	0.0	0.0	1.1 ± 0.1^{f}
1.5	0.2	2.6	0.1	0.3	0.0	3.1 ± 0.1^{e}
3	0.2	4.3	0.1	0.4	0.0	4.9 ± 0.2^{d}
4	0.5	6.0	0.0	0.5	0.1	7.0 ± 0.4^{c}
24	0.7	15.0	0.1	0.5	0.0	16.2 ± 1.2^{a}

* DP = degree of polymerization

**DH (%) = degree of hydrolysis (%) = total sugars weight at any given time/ initial dry weight of starch

Values with the same letter in the same column are not significantly different ($p < .05$)

Table 3.2 Percentage sugars (based on initial starch weight) in granular starches hydrolyzed by maltogenic amylase –B *

Hydrolysis time (h)	Waxy maize starch hydrolysates						DH (%) **
	DP- 1	DP- 2	DP- 3	DP- 4	DP- 5	DP- 6	
0.5	0.0	1.8	0.2	0.4	0.0	0.0	2.4 ± 0.5^{f}
1.5	0.1	2.9	0.3	1.1	0.4	0.2	4.9 ± 0.2^{de}
3	0.1	5.7	0.3	1.4	0.6	0.0	8.1 ± 0.2^{cd}
4	0.3	6.5	0.3	1.2	0.6	0.2	9.0 ± 0.6^{c}
24	1.4	10.5	0.2	0.6	0.4	0.1	13.2 ± 1.1^{b}

Hydrolysis time (h)	Normal wheat starch hydrolysates						DH (%) **
	DP- 1	DP- 2	DP- 3	DP- 4	DP- 5	DP- 6	
0.5	0.0	1.9	0.1	0.3	0.1	0.0	2.4 ± 0.3^{f}
1.5	0.1	3.2	0.2	0.4	0.1	0.0	3.9 ± 0.2^{ef}
3	0.2	5.1	0.1	0.6	0.1	0.0	6.1 ± 0.5^{d}
4	0.2	8.3	0.0	0.0	0.0	0.0	8.4 ± 0.7^{c}
24	1.7	20.6	0.2	0.6	0.1	0.0	23.3 ± 1.7^{a}

*DP = degree of polymerization

**DH (%) = degree of hydrolysis (%) = total sugars weight at any given time/ initial dry weight of starch

Values with the same letter in the same column are not significantly different ($p < .05$)

Table 3.3 Percentage sugars (based on initial starch weight) in starches swelled at 70°C, cooled to 62°C, and hydrolyzed by maltogenic amylase - A*

Hydrolysis time (h)	Waxy maize starch hydrolysates							DH (%) *
	DP-1	DP-2	DP-3	DP-4	DP-5	DP-6	DP-7	
0.5	0.2	23.2	2.7	6.1	2.2	1.1	0.1	36.0 ± 0.4^c
1.5	0.3	30.3	1.4	3.7	0.7	0.3	0.1	36.8 ± 1.1^c
3	0.8	33.7	1.2	1.5	0.4	0.0	0.0	37.4 ± 0.8^c
4	2.1	33.7	1.8	2.0	1.9	0.0	0.1	41.5 ± 0.6^b
24	4.0	38.8	1.4	1.2	0.4	0.0	0.0	45.6 ± 0.7^a

Hydrolysis time (h)	Normal wheat starch hydrolysates							DH (%) *
	DP-1	DP-2	DP-3	DP-4	DP-5	DP-6	DP-7	
0.5	0.8	21.0	1.2	2.6	0.7	0.0	0	26.1 ± 0.3^e
1.5	2.3	26.1	1.2	2.6	0.0	0.0	0	32.0 ± 1.5^d
3	3.3	30.3	1.2	1.9	0.0	0.0	0	36.4 ± 0.4^c
4	3.5	30.8	0.6	1.3	0.0	0.0	1.0	37.0 ± 0.2^c
24	5.7	38.7	0.6	0.6	0.0	0.0	0	45.9 ± 1.2^a

* DP = degree of polymerization

*DH (%) = degree of hydrolysis (%) = total sugars weight at any given time/ initial dry weight of starch

Values with the same letter in the same column are not significantly different (p < .05)

Table 3.4 Percentage sugars (based on initial starch weight) in starches swelled at 80°C, cooled to 62°C , and hydrolyzed by maltogenic amylase -A *

Hydrolysis time(h)	Waxy maize starch hydrolysates							DH (%) **
	DP- 1	DP- 2	DP-3	DP- 4	DP- 5	DP-6	DP-7	
0.5	0.1	25.0	4.0	13.3	1.7	1.1	0.0	45.2 ± 0.3^{c}
1.5	0.7	28.8	3.2	11.3	0.0	1.1	0.0	45.2 ± 0.9^{c}
3	1.5	34.3	2.4	10.2	0.9	1.1	0.0	49.5 ± 0.9^{b}
4	1.4	41.7	3.2	5.1	0.0	0.0	0.0	51.4 ± 0.7^{b}
24	2.9	51.5	0.8	1.0	0.0	0.0	0.0	56.1 ± 0.6^{a}

Hydrolysis time (h)	Normal wheat starch hydrolysates							DH (%) **
	DP- 1	DP- 2	DP-3	DP- 4	DP- 5	DP-6	DP-7	
0.5	0.4	28.8	2.4	6.1	0.0	0.0	0.0	37.4 ± 0.3^{d}
1.5	0.7	32.5	0.8	2.0	0.9	0.0	0.0	36.3 ± 0.4^{d}
3	2.9	31.0	1.6	2.0	0.0	0.0	0.0	$37.5 \pm .1.2^{d}$
4	1.5	33.4	0.8	2.0	0.0	0.0	0.0	37.7 ± 0.7^{d}
24	2.5	41.3	0.8	2.0	0.0	0.0	0.0	46.2 ± 0.4^{c}

*DP = degree of polymerization

**DH (%) = degree of hydrolysis (%) = total sugars weight at any given time/ initial dry weight of starch

Values with the same letter in the same column are not significantly different (p < .05)

Table 3.5 Percentage sugars (based on initial starch weight) in starches swelled at 90°C, cooled to 62°C, and hydrolyzed by maltogenic amylase -A*

| Hydrolysis time (h) | Waxy maize starch hydrolysates | | | | | | | DH (%) ** |
	DP- 1	DP- 2	DP-3	DP- 4	DP- 5	DP-6	DP-7	
0.5	0.4	42.3	1.6	2.8	0.0	0.0	0.0	47.4 ± 0.4^e
1.5	1.0	47.5	1.1	1.4	0.0	0.0	0.0	50.9 ± 0.1^d
3	1.9	54.5	0.5	1.4	0.0	0.0	0.0	58.1 ± 0.4^c
4	2.6	56.8	0.5	0.7	0.0	0.0	0.0	60.2 ± 0.2^b
24	3.0	68.4	0.0	0.0	0.0	0.0	0.0	65.9 ± 0.1^a

| Hydrolysis time (h) | Normal wheat starch hydrolysates | | | | | | | DH (%) ** |
	DP- 1	DP- 2	DP-3	DP- 4	DP- 5	DP-6	DP-7	
0.5	1.4	31.9	2.2	4.8	0.9	1.1	0.0	42.0 ± 0.2^f
1.5	1.6	41.7	1.6	2.3	0.0	0.0	0.0	47.2 ± 0.6^e
3	2.0	45.2	0.0	0.0	0.0	0.0	0.0	47.3 ± 0.5^e
4	2.7	47.5	0.5	0.0	0.0	0.0	0.0	49.8 ± 1.2^d
24	3.0	54.5	0.0	0.0	0.0	0.0	0.0	57.4 ± 0.7^c

*DP = degree of polymerization

**DH (%) = degree of hydrolysis (%) = total sugars weight at any given time/ initial dry weight of starch

Values with the same letter in the same column are not significantly different (p < .05)

Table 3.6 Percentage sugars (based on initial starch weight) in starches swelled at 70°C, cooled to 55°C, and hydrolyzed by maltogenic amylase -B *

Hydrolysis time (h)	Waxy maize starch hydrolysates							DH (%) **
	DP- 1	DP- 2	DP-3	DP- 4	DP- 5	DP-6	DP-7	
0.5	1.1	23.6	5.3	10.6	3.3	1.8	1.0	46.2 ± 0.4^d
1.5	3.4	31.2	3.2	7.1	2.5	0.9	0.0	48.1 ± 0.2^d
3	5.0	42.0	0.5	2.1	0.8	0.9	0.0	51.6 ± 1.4^c
4	6.5	47.1	0.5	0.7	0.8	0.0	0.0	55.3 ± 1.4^b
24	7.3	52.2	0.0	0.0	0.0	0.0	0.0	59.4 ± 1.8^a

Hydrolysis time (h)	Normal wheat starch hydrolysates							DH (%) **
	DP- 1	DP- 2	DP-3	DP- 4	DP- 5	DP-6	DP-7	
0.5	0.7	19.0	3.7	4.2	1.7	1.8	1.0	32.4 ± 2.1^e
1.5	2.0	26.6	2.1	2.8	0.8	0.0	0.0	34.4 ± 0.9^e
3	4.9	41.5	1.6	1.4	0.0	0.0	0.0	49.3 ± 0.6^{cd}
4	6.8	45.6	0.5	0.7	0.0	0.0	0.0	53.8 ± 0.4^{bc}
24	8.3	46.1	0.5	0.0	0.0	0.0	0.0	54.8 ± 1.5^{bc}

*DP = degree of polymerization

**DH (%) = degree of hydrolysis (%) = total sugars weight at any given time/ initial dry weight of starch

Values with the same letter in the same column are not significantly different (p < .05)

Table 3.7 Percentage sugars (based on initial starch weight) in starches swelled at 80°C, cooled to 55°C, and hydrolyzed by maltogenic amylase -B *

Hydrolysis time (h)	Waxy maize starch hydrolysates							DH (%) **
	DP- 1	DP- 2	DP-3	DP- 4	DP- 5	DP-6	DP-7	
0.5	1.4	24.6	5.4	7.8	3.4	2.2	1.2	46.0 ± 0.2^{de}
1.5	2.9	32.0	5.9	5.7	0.9	0.0	0.0	47.0 ± 1.1^{d}
3	5.6	44.1	1.5	1.4	0.0	0.0	0.0	52.7 ± 0.9^{c}
4	7.5	48.7	0.5	0.7	0.0	0.0	0.0	57.1 ± 1.2^{b}
24	9.4	51.5	0.0	0.0	0.0	0.0	0.0	60.9 ± 1.4^{a}

Hydrolysis time (h)	Normal wheat starch hydrolysates							DH (%) **
	DP- 1	DP- 2	DP-3	DP- 4	DP- 5	DP-6	DP-7	
0.5	3.8	27.8	5.4	5.0	1.7	1.1	0.0	44.3 ± 0.7^{e}
1.5	4.1	35.2	3.9	4.3	0.9	0.0	0.0	48.3 ± 0.6^{d}
3	5.6	39.0	3.0	2.8	0.0	0.0	0.0	50.5 ± 0.3^{d}
4	6.8	43.6	1.5	0.7	0.0	0.0	0.0	52.8 ± 0.8^{c}
24	8.3	47.8	0.0	0.0	0.0	0.0	0.0	56.1 ± 0.1^{b}

*DP = degree of polymerization

**DH (%) = degree of hydrolysis (%) = total sugars weight at any given time/ initial dry weight of starch

Values with the same letter in the same column are not significantly different (p < .05)

Table 3.8 Percentage sugars (based on initial starch weight) in starches swelled at 90°C, cooled to 55°C, and hydrolyzed by maltogenic amylase -B *

Hydrolysis time (h)	Waxy maize starch hydrolysates							DH (%) **
	DP- 1	DP- 2	DP-3	DP- 4	DP- 5	DP-6	DP-7	
0.5	1.8	29.8	5.7	6.2	2.3	1.7	0.8	48.5 ± 0.5^{ef}
1.5	4.3	42.2	3.7	3.7	0.7	0.0	0.0	54.6 ± 0.6^{d}
3	6.6	45.9	1.6	1.8	0.0	0.0	0.0	56.1 ± 0.3^{d}
4	7.7	51.8	0.8	1.2	0.0	0.0	0.0	61.6 ± 1.0^{b}
24	8.9	57.3	0.4	0.0	0.0	0.0	0.0	66.5 ± 0.7^{a}

Hydrolysis time (h)	Normal wheat starch hydrolysates							DH (%) **
	DP- 1	DP- 2	DP-3	DP- 4	DP- 5	DP-6	DP-7	
0.5	1.1	25.9	5.3	8.0	2.8	2.5	1.7	47.3 ± 0.6^{f}
1.5	6.1	38.5	2.5	3.0	0.0	0.0	0.0	50.0 ± 0.2^{e}
3	7.7	41.3	1.2	1.4	0.0	0.0	0.0	51.4 ± 1.0^{e}
4	7.9	45.4	1.2	0.0	0.0	0.0	0.0	54.3 ± 1.1^{d}
24	8.0	50.4	0.4	0.0	0.0	0.0	0.0	58.3 ± 0.7^{c}

*DP = degree of polymerization

**DH (%) = degree of hydrolysis (%) = total sugars weight at any given time/ initial dry weight of starch

Values with the same letter in the same column are not significantly different (p < .05)

Table 3.9 Gelatinization properties of native granular starches and products released by maltogenic amylase -A

Waxy maize starch	To	Tp	Tc	$\Delta H(J/g)$
control	59.9 ± 0.5^e	72.3 ± 0.6^f	91.9 ± 0.5^f	17.8 ± 0.8^a
0.5h	63.9 ± 0.3^{cd}	76.8 ± 0.7^{bd}	105.5 ± 0.8^a	17.6 ± 0.3^a
1.5h	62.4 ± 0.0^d	75.3 ± 0.7^e	98.1 ± 0.7^c	16.7 ± 0.4^{abc}
3h	64.5 ± 0.6^c	76.4 ± 0.3^{cde}	98.1 ± 0.2^c	16.8 ± 0.7^{ab}
4h	63.0 ± 0.6^{cd}	77.0 ± 0.3^{bc}	$98.8\pm0.3c$	15.5 ± 0.6^{bc}
24h	71.8 ± 0.0^a	82.0 ± 0.3^a	101.5 ± 0.2^b	8.7 ± 0.8^f

Normal wheat starch	Gelatinization peak				Amylose lipid peak			
	To	Tp	Tc	ΔH (J/g)	To	Tp	Tc	ΔH (J/g)
Control	57.6 ± 0.6^f	65.8 ± 0.1^h	86.3 ± 0.9^g	15.5 ± 0.5^{bc}	94.2 ± 0.8	103.9 ± 1.1	114.7 ± 1.0	1.7 ± 0.0
0.5h	59.4 ± 0.8^e	69.9 ± 0.6^g	94.5 ± 0.2^d	15.3 ± 0.3^c	97.2 ± 1.1	105.5 ± 1.2	116.1 ± 1.2	1.1 ± 0.1
1.5h	60.4 ± 0.6^e	69.6 ± 0.4^g	$92.5\pm0.5f$	13.8 ± 0.6^{cd}	98.4 ± 1.7	106.4 ± 1.7	116.9 ± 1.5	0.8 ± 0.3
3h	62.4 ± 0.4^d	72.0 ± 0.2^f	94.1 ± 0.6^{de}	12.7 ± 0.4^{de}	97.7 ± 0.8	106.2 ± 0.5	117.7 ± 0.2	1.2 ± 0.1
4h	61.8 ± 0.7^{de}	71.3 ± 0.5^f	94.7 ± 0.4^d	12.0 ± 0.2^e	95.6 ± 1.4	105.0 ± 1.1	117.7 ± 1.0	1.4 ± 0.0
24h	68.4 ± 0.8^b	77.9 ± 0.3^b	92.8 ± 0.7^{ef}	5.8 ± 0.1^g	101.0 ± 1.9	113.5 ± 1.7	119.9 ± 1.3	0.9 ± 0.1

Values with the same letter in the same column are not significantly different (p < .05)

Table 3.10 Gelatinization properties of native granular starches and products released by maltogenic amylase - B

Waxy maize starch	To	Tp	Tc	ΔH (J/g)
control	59.8±0.8[h]	72.0±0.0[c]	94.6±0.5[c]	18.5±0.3[a]
0.5h	64.3±0.7[bgf]	77.8±0.6[b]	100.4±0.7[b]	18.4±0.7[a]
1.5h	65.2±0.9[b]	77.6±0.6[b]	99.0±0.9[b]	17.4±0.5[a]
3h	64.9±0.8[bcd]	76.9±0.7[b]	102.1±0.8[a]	15.2±0.8[b]
4h	63.9±0.1[cde]	77.1±0.8[b]	102.9±0.7[a]	14.8±0.3[bc]
24h	68.0±0.0[a]	80.4±0.8[a]	93.6±0.2[ce]	6.9±0.0[f]

Normal wheat starch	Gelatinization peak				Amylose lipid peak			
	To	Tp	Tc	ΔH (J/g)	To	Tp	Tc	ΔH (J/g)
Control	56.1±0.3[i]	65.4±0.6[d]	87.0±0.3[g]	14.1±0.2[cd]	94.7±0.7	104.8±0.7	115.6±0.7	2.0±0.1
0.5h	62.9±0.8[dg]	72.9±0.4[c]	92.5±0.5[def]	12.7±0.5[d]	98.9±1.0	106.3±1.5	117.4±3.1	0.9±0.1
1.5h	63.7±0.1[cfd]	71.4±0.8[c]	92.9±0.6[def]	11.4±0.1[e]	99.2±1.3	107.0±1.0	115.8±0.2	0.5±0.1
3h	64.0±0.3[bd]	73.2±0.3[c]	94.1±0.4[cd]	11.3±0.5[e]	97.2±1.6	107.7±1.7	119.1±0.7	1.1±0.1
4h	63.9±0.7[befg]	71.7±0.8[c]	93.5±0.2[cf]	11.2±0.7[e]	97.9±0.4	106.2±1.1	119.6±1.0	0.9±0.1
24h	68.7±0.2[afg]	77.6±0.4[b]	92.6±0.6[d]	5.8±0.4[f]	104.0±0.9	112.2±1.4	122.2±0.6	1.4±0.1

Values with the same letter in the same column are not significantly different (p < .05)

Chapter 4 - Study of waxy maize starch hydrolyzed by maltogenic α-amylase in relation to its retrogradation

Bread staling (Chinachoti and Vodovotz 2000) is the biggest challenge faced by the baking industry (Sargent 2008) because it leads to huge economic losses (Gray and BeMiller 2003). Starch undergoes structural changes both during and after the baking process that greatly determine the quality of the final product. During cooling and storage of baked products, starch retrogrades, which leads to increased firmness. Long-term firmness of bread is due to the recrystallization of the gelatinized amylopectin (AP) network during storage, which results in increased crumb firmness and decreased crumb resilience (Goesaert et al. 2009; Gray and BeMiller 2003) thus leading to shorter shelf life.

Starch granules are semi-crystalline in nature and consist of amorphous and crystalline lamella within a radial arrangement of AP clusters. Amorphous lamellae contain a highly branched region, and crystalline lamellae consist of external AP chains (A chains and partial B chains) in the form of double helices (Hizukuri 1986; Zobel and Kulp 1996). During retrogradation, crystalline lamellae are involved in AP reordering, and branching initiates crystalline arrangement (Inouchi et al.1987). AP retrogradation depends on exterior chain length, with less retrogradation positively correlated with shorter chains (Inouchi et al.1987; Wursch and Gumy 1994). Several antistaling amylases are being used in the baking industry to prevent the formation of a recrystallized AP network by shortening AP side chains to prevent formation of double helices (Gerrard et al. 1997; Goesaert et al. 2009; Lundqvist et al. 2002). Production of low molecular weight dextrins upon enzymatic starch hydrolysis is also believed to inhibit bread staling by interfering with protein–protein, gluten–starch interactions (Hug Iten et al. 2003) and AP retrogradation (Defloor and Delcour 1999; Gerrard et al. 1997; Martin and Hoseney 1991). In addition to AP recrystallization, water migration from crumb to crust (Min et al. 1998; Sargent 2008) and cross-linking between the continuous protein matrix and discontinuous starch granules remnants (Hug Iten et al. 2003) are phenomena widely accepted to lead to bread firming.

Maltogenic α-amylase is an antistaling enzyme that is increasingly used in bakery products to improve their shelf life (Gerrard et al. 1997; Sargent 2008; Slade and Levine 1991).This enzyme is reported to act by shortening the AP side chains, thus preventing AP recrystallization and hindering water immobilization (Gray and BeMiller 2003; Goesaert et al.

2006). It exhibits both an endo and an exo action pattern, with a high degree of multiple attack action and with endo action increasing with increases in temperature (Bijttebier et al. 2007; Delcour et al. 2009). During baking, maltogenic α-amylase starts to act on starch granules when they begin to gelatinize, thus increasing their flexibility during storage. The resulting bread has a softer and more elastic crumb with an extended shelf life (Hug Iten et al. 2003). Mode of enzyme action and enzyme activity levels play an important role in determining the AP molecular structure. A higher degree of multiple attack action and higher enzyme activity levels used for porcine pancreatic α-amylase and *Bacillus Stearothermophilus* maltogenic α-amylase led to a 50% reduction in average chain length of outer AP chains, thus affecting the AP retrogradation to a greater extent; in contrast, the endo action of *Bacillus subtilis* and *Aspergillus oryzae* α-amylases, which are used at low enzyme activity levels, had only a limited impact on AP side chain distribution (Leman et al. 2006).

In this study, we subjected cooked waxy maize starch (WMS) to different degrees of hydrolysis (DH) by maltogenic amylase-A and examined the structure and retrogradation properties of the hydrolysates. We further related retrogradation properties to the chain length (CL) and molecular size (MS) distributions of debranched starch hydrolysates. Our objective was to study the action of maltogenic amylase on AP, relate the structure of the hydrolysates to their retrogradation properties, and determine how much hydrolysis was needed to prevent AP from retrogradation.

Materials and Methods

Materials

WMS (Amioca) from National Starch LLC (Bridgewater, NJ) was used for the study. Maltogenic amylase -A was provided by Caravan Ingredients (Lenexa, KS). Enzyme activity was assayed by quantifying the reducing sugars released from soluble starch (1.0% (w/v) solution) (St. Louis, MO) according to the Somogyi-Nelson method (Somogyi 1952) relative to a maltose standard curve. One enzyme unit is the amount of enzyme that releases 1 μmol of maltose/min at 62 °C for Novamyl and pH 5.0 (100 mM sodium acetate buffer containing 5 mM CaCl$_2$). Isoamylase (EC 3.2.1.68) was obtained from Hayashibara Biochemical Laboratories, Inc. (Okayama,Japan) and had an enzyme activity of 1.41 x 10^6 isoamylase activity units (IAU) /g (Cai and Shi 2010). All chemicals and reagents were of analytical grade.

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Enzymatic starch hydrolysis

Starch (1.8g db) was mixed with 90 ml of 100 mM sodium acetate buffer containing 5 mM $CaCl_2$ (pH 5.0), heated at 120 °C for about 40 min. in a pressure bottle (Ace Glass Incorporated, Vineland, NJ), and cooled to the optimum temperature of 62 °C for maltogenic amylase -A. Enzyme (9EU/g starch db) was added to the starch slurry to initiate the enzyme reaction. This enzyme level was selected based on previous studies (Leman et al. 2006; Splender et al. 1999). Starch was hydrolysed to 5, 10, 20, 30, 40, and 50% by taking aliquots at different time intervals. Enzyme was then denatured in a boiling water bath for 10 min. Starch hydrolysates were cooled to 25 °C and centrifuged at 8,000g for 10 min. Part of the supernatant was further analyzed for sugar profile analysis, and the rest of the samples were freeze-dried for further study.

Sugar profile analysis by high performance anion exchange chromatography (HPAEC-PAD)

Sugar profile analysis of the supernatants from WMS hydrolysates was done using an earlier cited procedure.21 Eluents were eluent A – 500 mM NaOH and eluent B – 150 mM NaOH with 0.5 M sodium acetate. The gradient program was: 85% eluent B for 0–0.4 min, 30% at 20 min, 25% at 30 min, 0% at 35 min, 0% at 40 min, 85% at 41 min, and 85% at 55 min. The separations were carried out at 25 °C with a flow rate of 1 ml/min. The concentration of the injected sample was 20 µg/ml in deionized water. Percentage DH was expressed as the total amount of sugars released at any given time based on initial dry weight of starch.

Molecular size distributions analysis by gel permeation chromatography (GPC)

MS distributions of starch hydrolysates were determined using GPC according to the previously cited procedure (Cai and Shi 2010).

Differential scanning calorimetry (DSC) of starch hydrolysates

We prepared 40% (w/w) starch hydrolysate samples in water. Samples were heated from 10 to 140 °C at 10 °C/min. The DSC pans were sealed and analyzed in duplicate using a Pyris-1 DSC (Perkin-Elmer, Norwalk, CT). An empty pan was used as a reference. The first scan was done on day 0, and the second scan was done on day 7 after storing the sample at 4 °C. The onset

(T_o), peak (T_p), and conclusion (T_c) temperatures and enthalpy (ΔH) were obtained from the DSC endotherm with DSC software (TA Instruments, New Castle, DE).

Debranching of starch hydrolysates

Freeze-dried starch samples were dissolved in 0.01 M acetic acid buffer (pH 4.0) with a starch: water ratio of 2:1. Starch solutions were cooked at 100 °C to ensure complete dissolution, then solutions were cooled to 50 °C. Isoamylase (1% based on dry weight of starch) was added. Debranching was done for 24 h followed by enzyme denaturation at 100 °C for 10 min. Debranching was done for another 24 h, and the enzyme was denatured by boiling samples at 100 °C for 10 min. The samples were cooled to room temperature before further analysis.

Chain length distribution analysis

Debranched AP samples were further analyzed for CL and MS distributions. Part of the samples was directly injected into HPAEC following debranching, and the remaining samples were freeze-dried for MS distribution analysis. For HPAEC, sample concentration of 1.5 mg/ml in 150 mM sodium hydroxide was prepared. Analysis was performed according to the previously cited procedure (Cai and Shi 2010). MS distributions were determined using GPC according to earlier cited procedure (Cai and Shi 2010).

Statistical Analysis

Each experiment was performed in duplicate. Data were analyzed by an analysis of variance (ANOVA) procedure with Tukey's studentized range (HSD) test using SPSS version 20.0 (IBM Corporation, Inc., Armonk, NY). Mean values from the duplicated experiments were reported. The least significant differences (LSD) for comparison of means were computed at $p < 0.05$.

Results and Discussion

Sugar profile analysis of starch hydrolysates

WMS hydrolysates were analyzed for sugars produced and to determine DH (%). Individual sugar percentages (based on initial starch weight) in the reaction mixture are given in Table 3.1. Sugars up to degree of polymerization (DP) 6 were formed, and their concentrations

continued to increase with increase in hydrolysis time with the formation of DP 7 toward the end of 30 min. Maltose was the predominant sugar produced. The rate of formation of maltose was significantly higher than other sugars. The maltose: glucose ratio increased with an increase in DH (%). Increasing the hydrolysis time beyond 30 min resulted in a decrease in maltose: glucose ratio and breakdown of higher DP sugars into glucose and maltose via secondary reactions (previous data). This result indicates that the exo action of enzyme seen during earlier hydrolysis stages switched to an endo action during later stages. A change in action pattern of maltogenic amylase from exo to endo with increase in hydrolysis time (Delcour et al. 2009) and increase in temperature (Gray and BeMiller 2003) was reported in earlier studies, as was the production of sugars up to DP 7–9, which were further hydrolysed to maltose by this enzyme (Bijttebier et al. 2010; Splender et al. 1999).

Molecular size distributions of starch hydrolyzates

Starch hydrolysates were analyzed for MS distributions using GPC. High MS fraction hydrolysates decreased, whereas hydrolysates with low MS fraction increased with an increase in percentage DH (Figure 3.1). In addition to substrate structure, substrate concentration and enzyme activity level plays an important role in determining the enzyme action. We observed an insignificant shift to low MS regions for 5% gelatinized WMS hydrolysed by maltogenic amylase (5 EU/g starch) until DH reached 10% (Delcour et al. 2009). A change in action pattern of maltgenic amylase from exo to endo as the hydrolysis time increased was reported earlier (Christophersen et al. 1998), but action changed from exo to endo as the temperature reached close to gelatinization temperature in another study (Gray and BeMiller 2003).

Thermal and retrogradation properties

Retrogradation properties of starch hydrolysates after 7 days of storage at 4 °C are shown in Table 3.2. Starch hydrolysates showed no endothermic peak during the initial scan before storage (day 0) because the starch was completely cooked prior to hydrolysis. WMS showed significant retrogradation after 7 days of storage at 4 °C. AP retrogradation enthalpy decreased to 0.8 J/g for 20% hydrolysed starch. As DH increased, the extent of retrogradation decreased, with no retrogradation observed for starches hydrolyzed to greater than 20% DH. We believe the shortened outer chains inhibited formation of double helices and prevented AP recrystallization. Another reason for this result might be the increase in low MW dextrins with an increase in DH,

which interferes with AP recrystallization. These results are consistent with previous studies conducted on bread crumbs (Gerrard et al. 1997; Gray and BeMiller 2003). Wider enthalpy endotherm for control was due to the presence of large and imperfect crystals during retrogradation (Beier et al.2000; Cooke and Gidley 1992). Upon enzyme digestion, these crystals became more perfect, so we observed narrowing of the endotherms with increase in percentage DH. Retrogradation tendency of AP depends on its CL distribution (Cooke and Gidley 1992; Yuan et al. 1993), with retrogradation rate having a positive correlation with mole fraction of unit chains of DP 14–24 and a negative correlation with mole fraction of unit chains of DP 6–11 (Shi and Sieb 1992; Yuan et al. 1993). Using model compounds, we observed no retrogradation when DP was less than 10 (Gidley and Bulpin 1989; Shi and Sieb 1995); however, in the presence of longer chains, chains as short as DP 6 can cocrystallize (Pfannemuller 1987).

Chain length distributions of debranched waxy maize starch hydrolysates

WMS hydrolysates were debranched with isoamylase to determine the fine structure of residual AP. HPAEC-PAD was used to characterize the individual components of debranched AP (Figure 3.3 a–f). At low percentage DH, not much significant difference was observed compared with the control, but with an increase in DH, the unit chains with DP<10 increased with a corresponding decrease in higher DP unit chains. These results are in line with previous findings (Christophersen et al. 1998). Higher relative amounts of CL with DP≤9 supports the complete suppression of retrogradation with a percentage DH≥20 because of a decreased tendency of these short chains to participate in double helical formation. Double helices formed by adjacent AP chains give rise to crystalline lamellae, whereas branching points and amylose constitute amorphous lamellae (Gidley and Bulpin 1987).

With an increase in hydrolysis, unit chains of DP 17–24 and DP≥24 decreased, whereas unit chains of DP≤11 increased (Table 3.3). Chains with DP 10–11 and DP 12–16 also increased, but only slightly compared with the control. Relating the retrogradation data with CL distributions, we can conclude that the higher the proportion of unit chains with DP≤9, the lower the retrogradation rate. A minimum CL of DP 10 is required for formation of stable double helices (Gidley and Bulpin 1989; Shi and Sieb 1995). The shortest A chains with a DP of 5–10 cannot participate in stable double helices formation and disrupt the formation of an ordered crystalline structure and negatively affect the perfection of AP crystalline structure (Yao et al.

2003). Outer AP chains (mainly A and B1) greatly influenceAP's molecular reorganization and aggregation during storage (Leman et al. 2006). The net decrease in proportion of unit chains with DP 14–24 could be another reason for reduced AP retrogradation (Yuan et al. 1993). The large decrease we observed for outer AP chains is in line with previous findings suggesting that the exo action of this enzyme (Splender et al. 1999; Srichuwong and Jane 2007) is similar to β-amylase, which removes the external chains from AP (Zhang et al. 2006). Compared with conventional α-amylase, which only cleaves internal starch chains and results in reduced MS without having much effect on outer branches of AP, maltogenic α-amylase greatly degrades AP side chains and thus prevents crystalline junction zone formation (Gray and BeMiller 2003). High relative amounts of very short AP chains were correlated with reduced AP retrogradation (Inouchi et al. 1987; Outtrup and Norman 1984).

Molecular size distributions of debranched waxy maize starch hydrolysates

MS distributions of debranched samples and starch hydrolysates are shown in Figure 3.4. Debranched samples showed four distinct peaks in MS distribution with higher proportions of short unit chains, which increased with an increase in DH. With an increase in percentage DH, debranched starches showed a lower proportion of large molecules and a higher proportion of small molecules, which is in line with CL distributions analysis results (Table 3).

Conclusions

Sugar profile analysis of starch hydrolysates showed the formation of higher DP sugars during the course of hydrolysis with maltose constituting a major fraction in the reaction mixture. Maltogenic amylase -A was able to shorten the outer CL of AP and permanently prevent it from retrogradation after 20% hydrolysis. AP retrograded to one-third after 10% hydrolysis compared with unhydrolysed AP. We found a positive correlation was found with level of unit chains (DP≤9) and retrogradation inhibition with an increase in DH (%). These results indicate the antistaling effect of this enzyme even at limited starch hydrolysis (20%).

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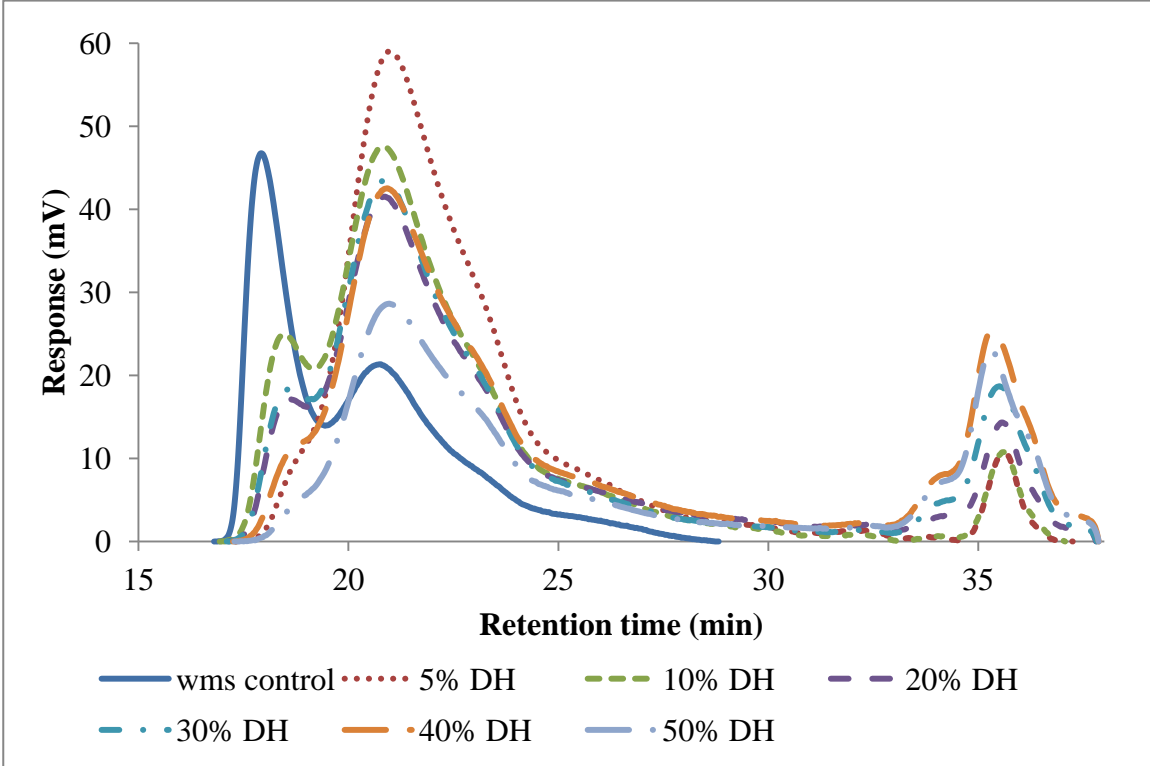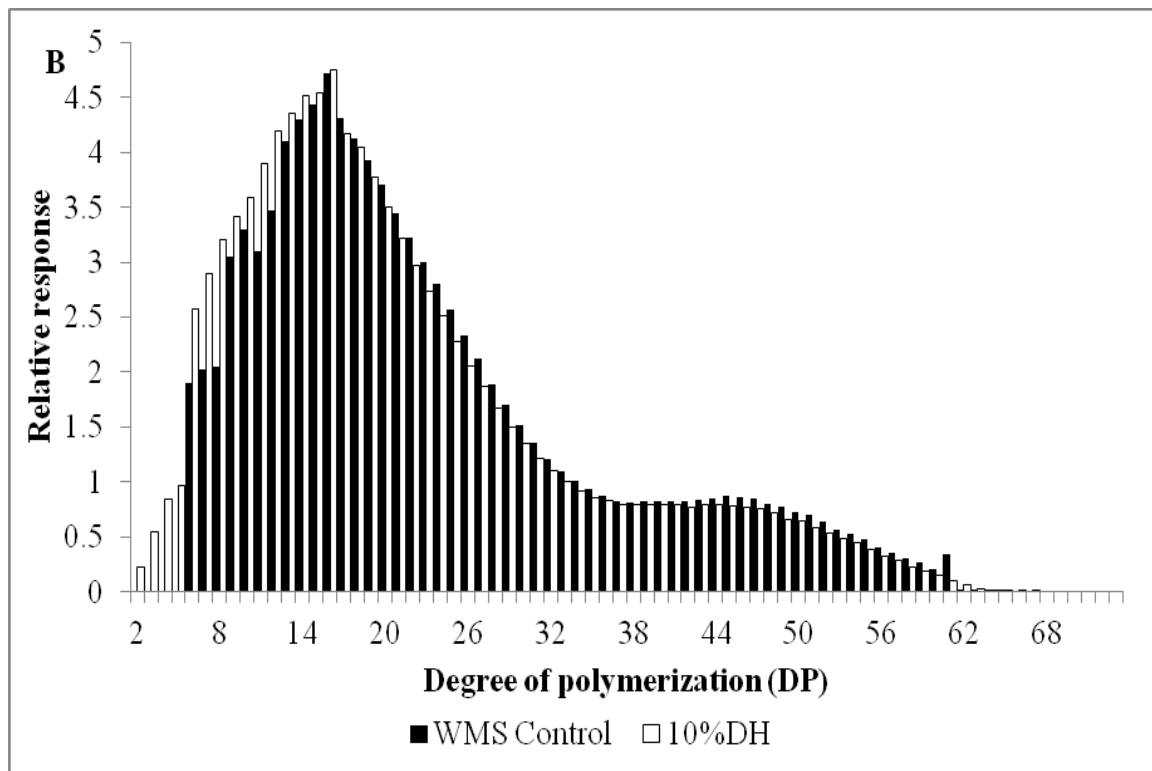**Figure 4.1 Molecular size distributions of waxy maize starch hydrolysates**
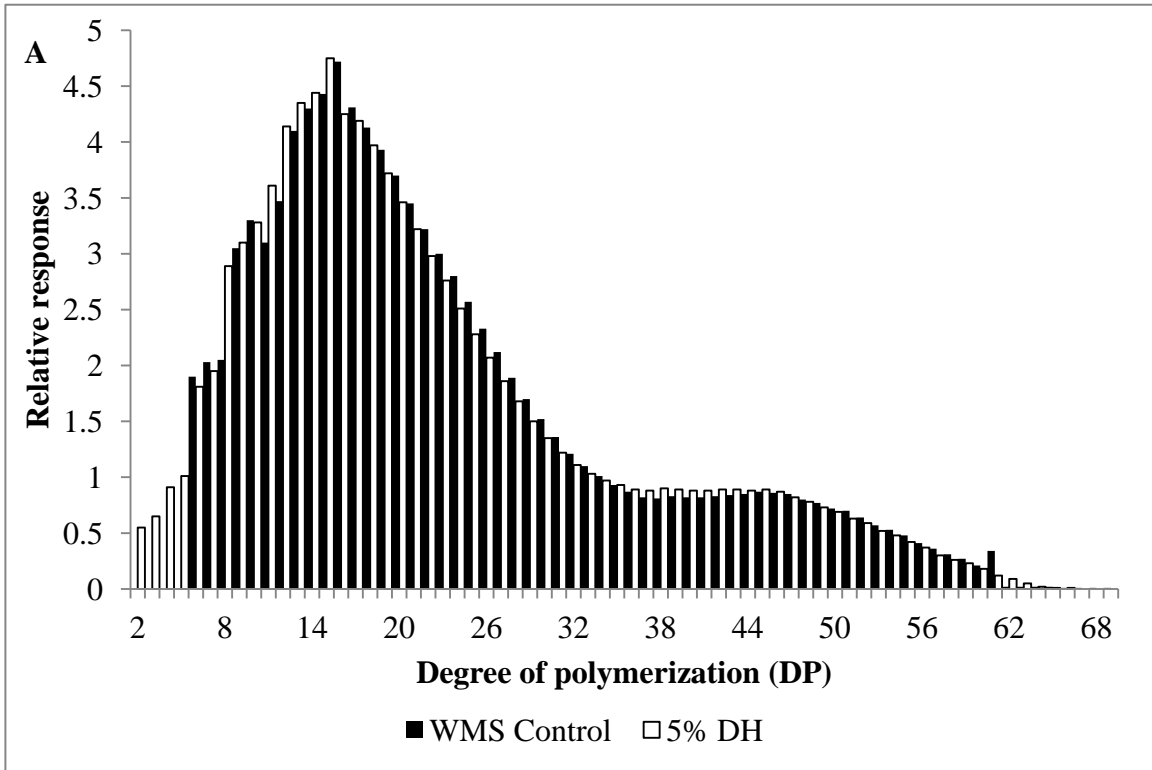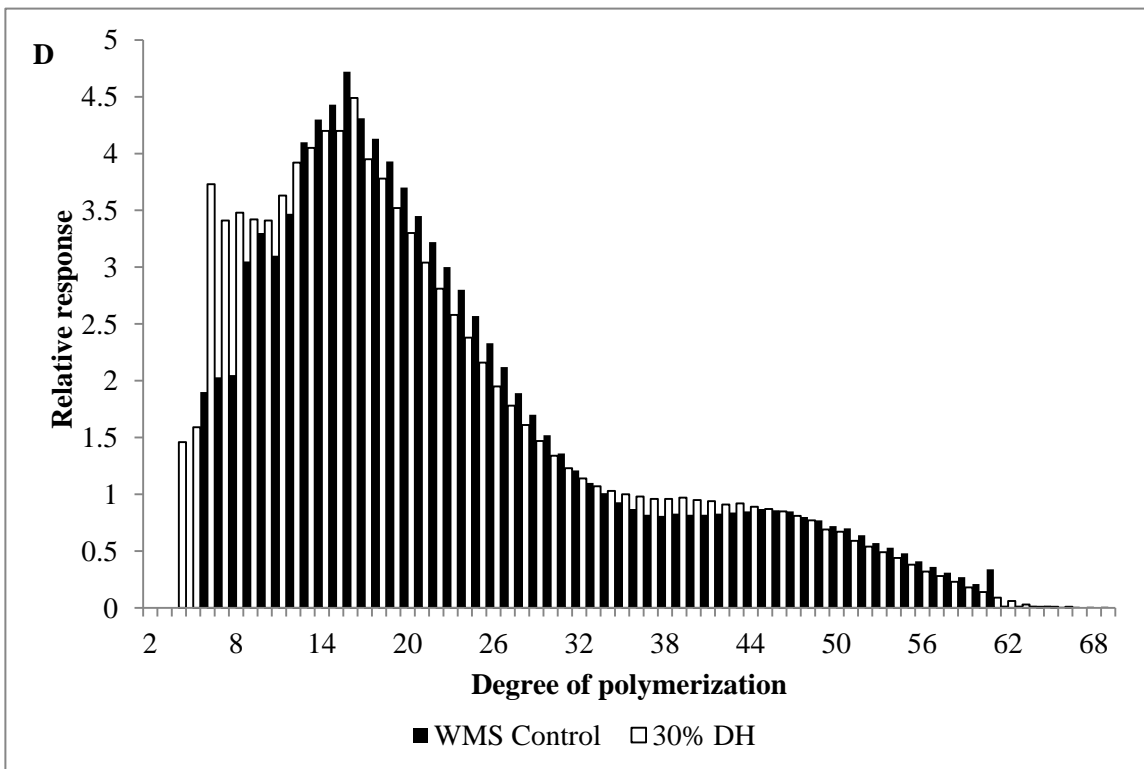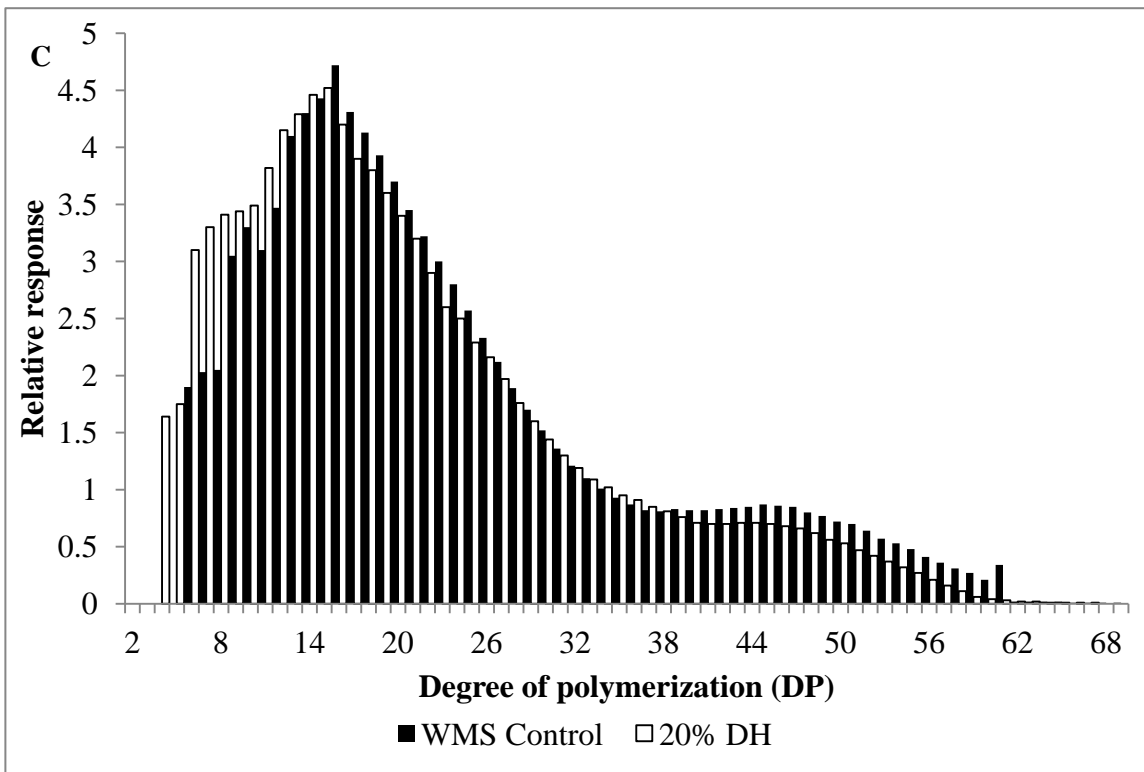
Figure 4.2 Chain length distributions of waxy maize starch compared to hydrolysates after (a) 5%, (b) 10%, (c) 20%, (d) 30%, (e) 40%, and (f) 50% hydrolysis
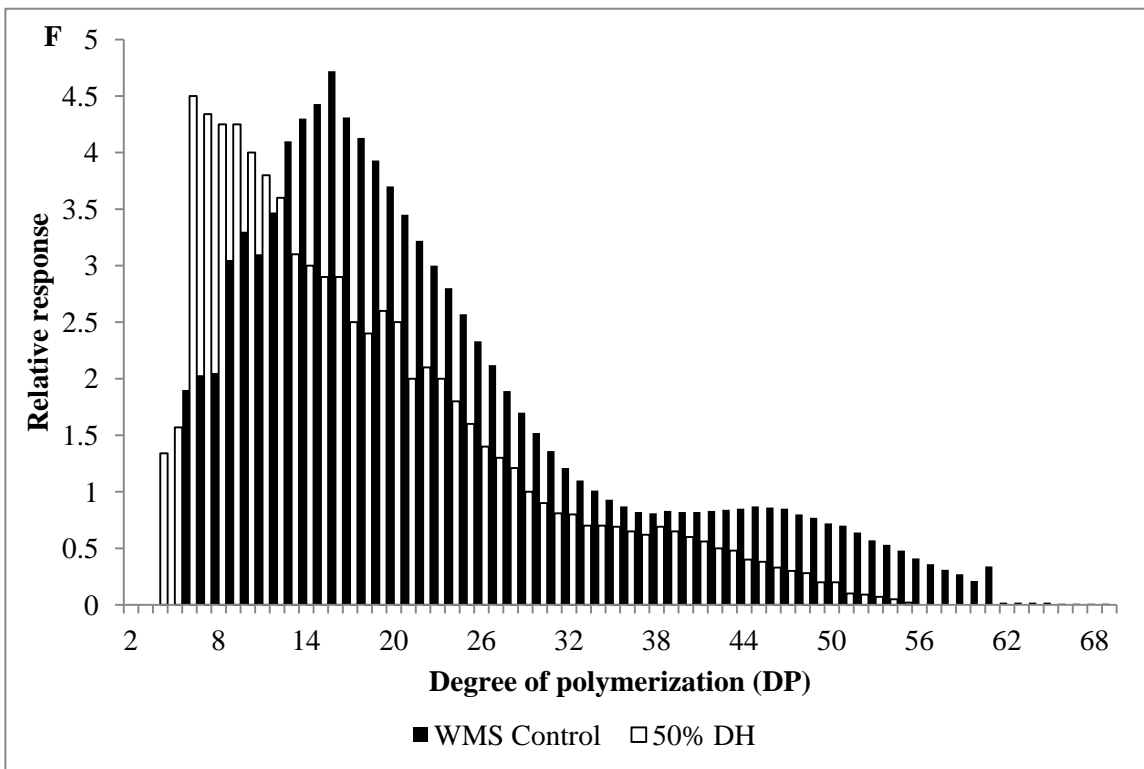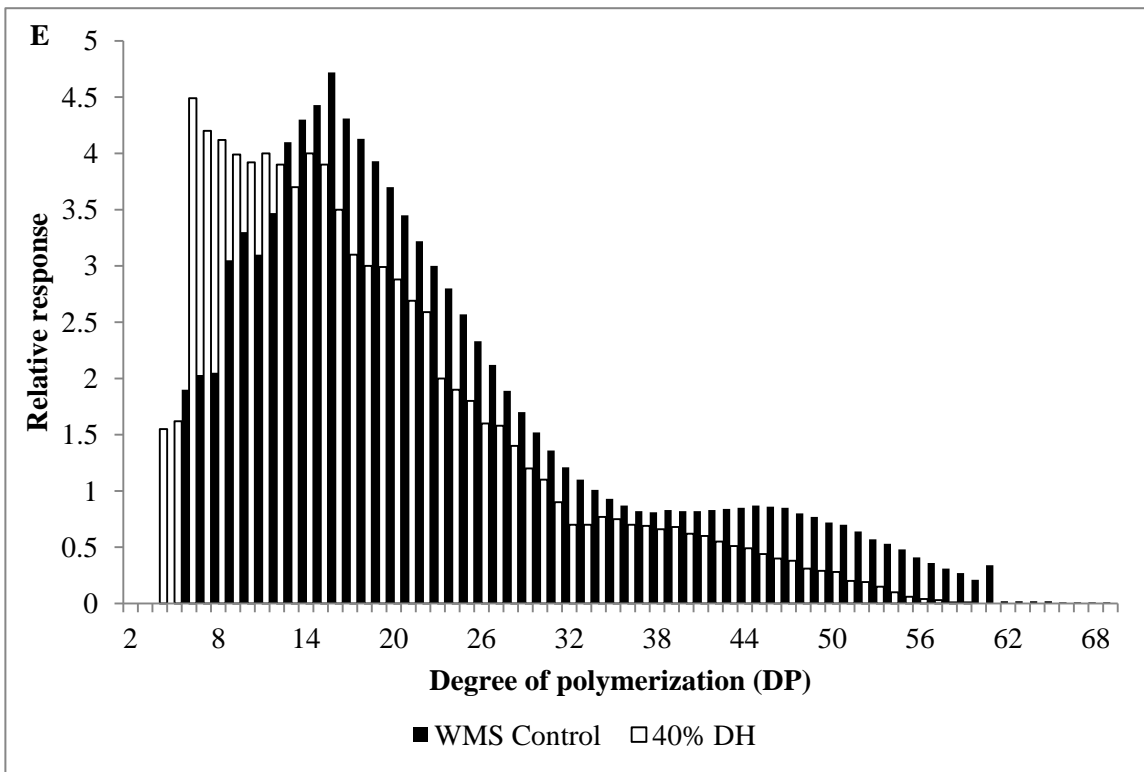
Figure 4.3 Side-chain distributions of residual amylopectin structure after enzyme hydrolysis
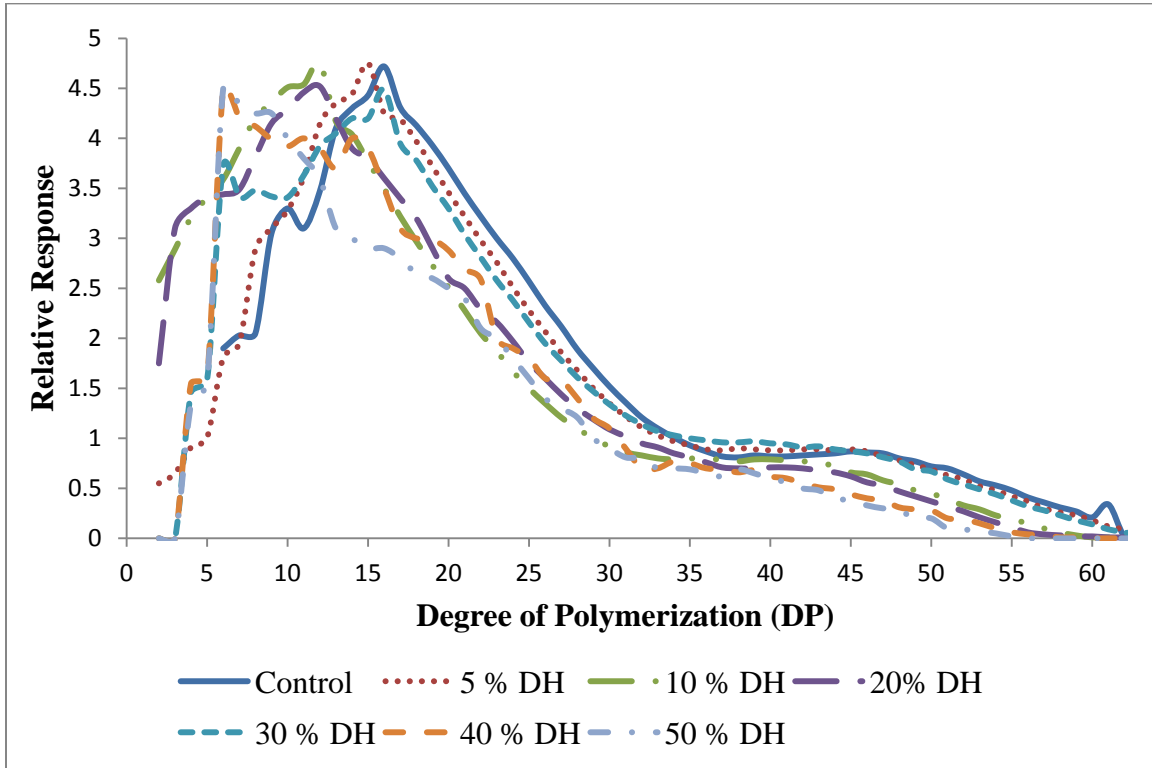

Figure 4.4 Molecular size distributions of debranched waxy maize starch hydrolysates
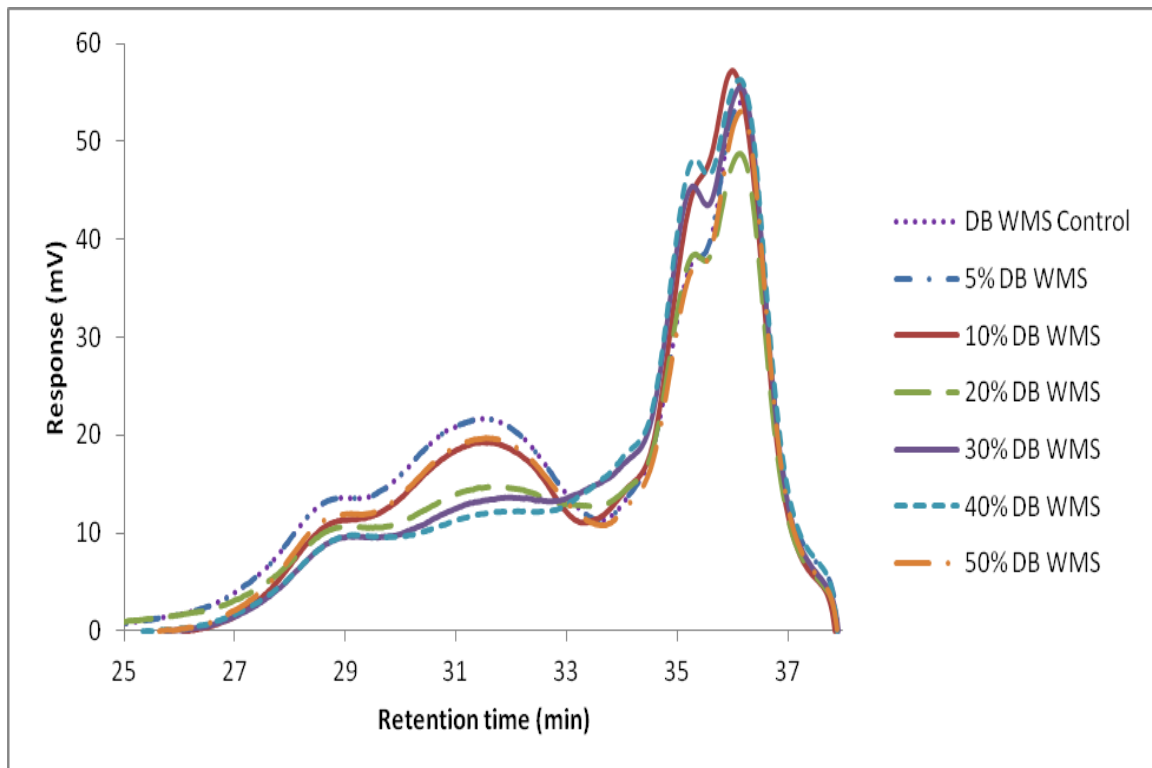
Table 4.1 Composition of sugars in waxy maize starch hydrolysates after different degrees of hydrolysis

Sample DH (%)*	Degree of polymerization						
	1	2	3	4	5	6	7
5	0.5	3.3	0.3	0.3	0.1	0.0	0.0
10	0.6	9.3	0.6	0.6	0.1	0.1	0.0
20	0.7	16.6	0.9	2.4	0.2	0.1	0.0
30	0.8	26.1	1.1	3.6	0.3	0.1	0.0
40	0.8	33.6	1.3	4.9	0.4	0.2	0.0
50	1.1	40.7	1.7	5.6	0.4	0.2	0.0

[a] DH (%) (degree of hydrolysis) was calculated as total amount of sugars released at any given time based on initial starch weight.

Table 4.2 Retrogradation properties of waxy maize starch hydrolysates (after storage at 4 °C for seven days) as determined by differential scanning calorimetry

Sample	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)
Control	38.7 ± 0.9^c	54.8 ± 1.2^a	79.9 ± 1.6^a	9.1 ± 0.8^a
5 % DH	38.4 ± 0.3^c	56.6 ± 0.7^a	78.8 ± 0.2^a	7.1 ± 0.2^b
10 % DH	41.6 ± 0.7^b	57.4 ± 0.1^a	73.1 ± 0.3^b	3.2 ± 0.2^c
20% DH	46.0 ± 0.2^a	59.8 ± 0.5^a	73.6 ± 0.6^b	0.8 ± 0.1^d
30% DH	-	-	-	-
40% DH	-	-	-	-
50% DH	-	-	-	-

Values with the same letter in the same column are not significantly different ($p < 0.05$)

Table 4.3 Level (%) of unit chains in debranched waxy maize starch hydrolysates

Sample (%DH)*	Degree of polymerization				
	DP <9	9-11	12-16	17-24	≥25
0	5.98	9.45	21.02	28.54	35.04
5	9.68	10.01	22.00	26.81	33.04
10	10.27	10.90	22.36	26.32	30.15
20	12.20	10.75	21.62	25.9	29.92
30	13.67	10.46	20.86	25.36	29.72
40	17.98	16.91	20.00	21.15	22.89
50	20.45	18.78	18.65	19.99	20.28

[a] DH (%) (degree of hydrolysis) was calculated as total amount of sugars released at any given time based on initial starch weight.

Chapter 5 - Final Conclusions

Differences in action patterns of both enzymes were evidenced by differences in oligosaccharides compositions and MS distributions of hydrolysis products released from dispersed, swelled and granular starches. For any given type of starch, hydrolysis time and temperature, maltogenic amylase -B achieved a higher DH and MS reduction than maltogenic amylase -A. This might be due to its higher endo activity displayed on non reducing ends blocked compound, p-nitrophenol maltoheptaoside. This indicated that maltogenic amylase -B can cause greater weakening of starch network leading to improved bread crumb resilience.

Morphology study of granular starch hydrolysates showed that both enzymes followed a heterogenous attack with damage to few granules with most granules still largely intact. For WMS, maltogenic amylase -A caused pinholes formation which widened up during course of hydrolysis while maltogenic amylase -B caused more of surface erosion with fewer pinholes. NWS showed extensive damage to A granules (10-40μm) with deep cavities formation during later stages by both the enzymes. A decrease in gelatinization enthalpy and increased gelatinization temperature was observed with time due to formation of stable crystallites. A decrease in crystallinity as was observed by XRD study suggesting hydrolysis of both amorphous and crystalline regions by these enzymes. However, their action pattern was quite different on both starches.

Relationship between amylopectin retrogradation and DH (%) indicated that at limited hydrolysis (20%), maltogenic amylase -A exhibited antistaling effect on starch. A positive correlation was established between the level of unit chains (DP ≤ 9) and retrogradation inhibition with increase in DH (%). This means that maltogenic amylase-A can serve as a powerful antistaling agent even at limited starch hydrolysis.

Bread being a very complex system consisting of different ingredients, future research could be directed towards understanding enzyme behavior under actual baking conditions.