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Reaction pattern of a novel thermostable α-amylase

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Abstract

A novel thermostable α-amylase, D45 was studied for its reaction pattern on starch hydrolysis. Fine structures of the dextrins and oligosaccharides produced by D45 were determined and compared with those produced by other thermostable α-amylases, Termamyl®LC (LC) and Termamyl®SC (SC). Waxy maize starch dispersion was hydrolyzed with LC, SC and D45 at different concentrations to obtain hydrolysates with the same dextrose equivalent value (DE). At DE ~13, molecular weight distribution of dextrins produced by D45 displayed a mono-distribution with a peak centered at degree of polymerization (DP) of 7, whereas LC and SC hydrolysates displayed a bimodal-distribution of the molecular weight profiles with one peak centered at DP 5 and the other at DP 34. Thin-layer chromatograms (TLC) showed that DP 2, 3, 5, 6 and 7 were the primary oligosaccharides produced in LC hydrolysate, DP 4–7 in SC hydrolysate, and DP 6–9 in D45 hydrolysate. Comparison of the decrease in the blue color of amylose-iodine complex at 620 nm (blue value) with the increase in reducing value for the hydrolysis of amylose by LC, SC and D45 showed that for an equivalent decrease in blue value, LC and SC produced a higher percentage of reducing sugar than did D45. The results suggest that D45 has a greater degree of random attack (multichain) reaction, whereas LC and SC have more multiple-attack reactions.

Keywords: Reaction pattern; Thermostable α-amylase; Waxy maize starch; Liquefying enzyme

1. Introduction

Alpha-amylase (EC 3.2.1.1) is an endo-1, 4-α-D-glucohydrolitic enzyme, which occurs widely in microorganisms, plants, and animals, and has found applications in numerous industries. One of its major applications is for liquefaction of starch in the production of glucose and high-fructose corn syrup. The typical process for making high-fructose corn-syrup involves many steps, including wet-milling corn starch, liquefying the starch slurry to dextrins at pH ~6 and 95–105 °C using a thermostable α-amylase, saccharifying the dextrins to glucose with amyloglucosidase at pH 4.5, and finally, converting glucose to fructose with glucose isomerase at pH 7 (Reilly, 1985; Simms, 1985; Van der Maarel, Van der Veen, Uitdehaag, & Leemhuis, 2002). Commonly used thermostable α-amylases is from *Bacillus licheniformis* and *Bacillus stearothermophilus.* One drawback of these commercial α-amylases is their reduced activity at low pH (Van der Maarel et al., 2002). As a consequence, pH adjustment is needed to raise the pH of the starch slurry from its natural pH 4.5 to a value where these amylases are effective, typically around pH 5.5 before the liquefaction process. Further pH adjustment is needed to bring the pH to 4.5 (the optimum pH of amyloglucosidase) before saccharification. Moreover, these amylases require calcium (Ca^{++}) for their thermal stability (Van der Maarel et al., 2002). Because removal of calcium and salts generated during pH adjustment is a costly process, thermostable α-amylases with lower pH optima and no requirement for added calcium are of great interest to the industry.

A novel α-amylase, D45 developed by Diversa Corporation (San Diego, CA) using proprietary protein evolution methods was reported to have characteristics of pH and temperature tolerance well suited to the commercial starch liquefaction process (Richardson, Tan, Frey, Callen, Cabell and Lam, 2002). The enzyme is extremely stable at high temperature without the addition of calcium and was optimally active at pH 4.5 when tested under industrial corn wet milling conditions (Richardson et al., 2002). The HPLC profile of D45-liquefied starch showed similar products, with slightly higher molecular-weight oligomers, to the products generated by a commercial α-amylase from *B. licheniformis*. Moreover, after saccharifying with a commercial amyloglucosidase, both

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glucose syrups contained the same level of glucose (Richardson et al., 2002).

The objectives of this study were to investigate the reaction mechanism of the novel α-amylase, D45, during starch hydrolysis and to characterize fine structures of the oligosaccharides generated by D45 and by commercially available thermostable α-amylases from *B. licheniformis* (Termamyl®LC) and from *B. stearothermophilus* (Termamyl®SC) (Novozymes, Bagsvaerd, Denmark).

2. Experimental

2.1. Materials

Waxy maize starch was a gift from Cerestar, USA (Hammond, IN). Termamyl®LC (LC) and Termamyl®SC (SC) are the products of Novozymes (Bagsvaerd, Denmark). Pullulanase from *Bacillus acidopullulyticus* (Promozyme®400L) and potato amylose were purchased from Sigma Chemical Co. (St. Louis, MO). Proteinase K (Fungal) was purchased from Invitrogen Co. (Carlsbad, CA). All other chemicals were of analytical grade and were used without further treatment.

Amylase D45 was provided by Diversa Corp. (San Diego, CA). D45 is a mono-component highly thermostable amylase enzyme recovered from a controlled fermentation of a pure culture of *Pseudomonas fluorescens*. Recovery of D45 amylase from *P. fluorescens* fermentations involves treatment at high temperature, adjustment to high alkaline pH and sequential filtration steps, all of which are designed to remove extraneous material and maximize recovery of D45. As a consequence, the D45 product is very pure containing little protein other than D45 amylase (SDS-PAGE data) and no other detectable amylolytic activities (data not shown).

2.2. α-Amylase hydrolysis of waxy maize starch

Waxy maize starch dispersion (1%, w/v) was prepared, following the method of Yoo & Jane (2002), by dispersing the starch in a 90% DMSO solution with mechanical stir in a boiling water bath, precipitating the starch with ethanol, and redispersing it in a buffer solution (0.02 M acetate buffer, pH 5.6, with 20 ppm of Ca^{++} for LC and SC, and 0.02 M phosphate buffer, pH 6, with 20 ppm of Ca^{++} for D45) in a boiling water bath. The starch dispersion was hydrolyzed with amylase (3.1, 12.5 and 46.0 units[1]/g starch for LC, SC and D45, respectively) at 70 °C for 5, 15, 30 and 60 min. The enzyme reaction was stopped by adjusting the pH of the hydrolysate to pH 12 with 1 N NaOH. The reducing sugar and total carbohydrate contents of the hydrolysates were determined by using the Somogyi-Nelson method and Phenol-sulfuric method, respectively (Jane, Shen, and Aguilar, 1992). Dextrose Equivalent (DE) value was calculated as DE = [reducing sugar]/[total carbohydrate] × 100.

2.3. Molecular weight distributions of products in enzyme hydrolysates

The molecular weight distribution of products in the α-amylase hydrolysates was determined by using high-performance size-exclusion chromatography equipped with multi-angle laser-light scattering and refractive index detectors (HPSEC-MALLS-RI), following the method of McPherson & Jane (2000); Yoo et al. (2002). A Shodex SB-803 HQ analytical column with a Shodex OH pack KB-G guard column (Showa Denko, Tokyo, Japan) was used for the analysis. Average molecular weights by weight (M_w) and by number (M_n) were determined from the chromatograms and were compared with those obtained by chemical methods ([total carbohydrate]/[reducing sugar]).

2.4. Thin-layer chromatography (TLC)

Oligosaccharides present in the α-amylase hydrolysates were analyzed by using thin-layer chromatography, following the method of Robyt & Mukerjea (1994). The samples were spotted onto a 20 × 20 cm Whatman K5 TLC plate (Fisher Scientific, Chicago, IL), and the plate was repeatedly irrigated (2–4 times) with a solvent mixture of acetonitrile: ethyl acetate: 1-propanol: water (85: 20: 50: 50, by volume) at 25 °C. The carbohydrates on the TLC plate were detected by dipping the dried plate into a methanol solution containing *N*-(1-naphthyl) ethylenediamine (0.3% w/v) and sulfuric acid (5% v/v), followed by heating at 120 °C for 10 min. Carbohydrates were quantified on the TLC plate by measuring staining intensity using a Scion Image-Release Beta 4.0.2 Program (Scion Corporation, Frederick, MD).

2.5. Reaction pattern of α-amylase

Reaction patterns for LC, SC and D45 were determined by comparing the decrease in blue color of amylose-iodine complex (blue value) with the increase in reducing value after hydrolysis of amylose, following the method of Robyt & French (1967) with modification. Amylose (12.4 mg) was dissolved in 90% DMSO (0.2 ml) with mechanical stir in a boiling water bath for 1 h. 1.2 ml of 0.2 M buffer solution (acetate buffer, pH 5.6 for LC and SC, and phosphate buffer, pH 6 for D45) with Ca^{++} (200 ppm) added to the dispersion before diluting with distilled water to 12 ml. The amylose dispersion was then hydrolyzed with amylases (1.5, 1.3 and 13.2 units/mg amylose for LC, SC and D45, respectively) at 70 °C for various time periods. Enzyme reactions were stopped by adjusting the pH of the hydrolysate to 12 with 1 N NaOH. The decrease in amylose-iodine complex color at 620 nm (blue value) and the increase in reducing sugar as reaction proceeded were measured by using iodine and the alkaline ferricyanide method, respectively (Robyt and French, 1967).

[1] Amylase specific activity was expressed as units per mg protein where an enzyme unit is the amount of enzyme producing 1 μmole of maltose per minute at 70 °C.

2.6. Structure of α-limit dextrins

Alpha-limit dextrins were prepared by hydrolyzing a dispersion of waxy maize starch (0.5% w/v) using 100 units/g starch of LC, SC or D45 at 70 °C until the reducing sugar value had plateaued. Inactivation of α-amylase was needed in advance to prevent subsequent hydrolysis by α-amylase of linear chains produced when α-limit dextrins were debranched by pullulanase. Several methods were tested, including adjustment of pH to 2 or to 12 while heating in a boiling water bath, but the thermostable α-amylases in the hydrolysates regained their activity once the pH was adjusted back to 4.5 (data not shown). The method of choice used proteinase K to hydrolyze and inactivate the α-amylase proteins. The α-amylase hydrolysates were treated with proteinase K (910 units/unit of α-amylase) at pH 8, 65 °C for 3 h, and the proteinase K was then inactivated by boiling the mixture for 30 min. To confirm that the amylases had been inactivated by proteolysis, we prepared waxy maize starch dispersions (0.5%) and incubated them at 70 °C with α-amylases that had been previously treated with proteinase K at 65 °C for 3 h (using the same condition as above) and then boiled for 30 min. Reducing sugar content of the enzyme-treated waxy maize starch dispersion was determined after varying time intervals.

For structure determination, the α-limit dextrins were debranched by incubating with 50 units pullulanase/g starch at 55 °C for 15 h in an acetate buffer (pH 4.5, 20 mM). The debranched samples were then analyzed for DP values by the chemical methods described earlier, and the oligosaccharide products were analyzed by using TLC.

3. Results and discussion

3.1. α-Amylase hydrolysis of waxy maize starch

DE values obtained for waxy maize starch hydrolyzed with LC, SC and D45 for various time intervals, 15, 30, and 60 min., are presented in Table 1. The enzyme concentrations were selected to produce hydrolysates with similar DE values of 8–10, which are typical DE values for liquefied starch used commercially for the production of glucose syrup (Reilly, 1985; Van der Maarel et al., 2002). Molecular weight distributions of products in starch hydrolysates with different DE values are shown in Fig. 1(A)–(C). The D45 hydrolysate displayed a mono-distribution of molecular weight, whereas LC and SC hydrolysates displayed a bimodal-distribution of product molecular weight (Fig. 1(A)–(C)). This bimodal molecular weight distribution has previously been reported for the hydrolysis of potato amylopectin by *B. licheniformis* (Marchal et al., 1999) and *B. subtilis* (Heitmann, Wenzig, & Mersmann, 1997) α-amylases. At early stages of hydrolysis, a D45 hydrolysate with DE 6.5 exhibited a peak in the molecular weight distribution of products centered at DP 16 (Fig. 1(C)). No product with DP larger than 66 was observed (Fig. 1(C)). As hydrolysis progressed, the DE value of the D45 hydrolysate increased to 12 and the peak in the molecular weight distribution shifted to DP 7 (Fig. 1(C)). The LC hydrolysate with DE 4.3 displayed two peaks, one at DP 152 and a second peak at DP 6 (Fig. 1(A)). The SC hydrolysate with DE 4.5 also showed two peaks at DP 113 and DP 5 (Fig. 1(B)). As LC and SC hydrolysis progressed to DE 14.7 and 12.2, respectively, the elution peaks of products in both enzyme hydrolysates showed gradual shifts to lower molecular weights and an increase in peak area of the small molecular weight fraction, along with a decrease in peak area of the larger molecular weight fraction (Figs. 1(A) and (B)). The peaks obtained from LC and SC hydrolysates after 60 min incubation were centered at approximately DP 34 and DP 5, for large and small molecular weight products, respectively.

The average molecular weight and DP by weight (M_w) and by number (M_n) obtained from HPSEC profiles, the polydispersity (M_w/M_n), and the DE values of the LC, SC

Table 1
Average molecular weight, polydispersity, DP, and DE values of α-amylolytic products of waxy maize starch separated by using HPSEC

α-Amylase	Hydrolysis time (min)	Average molecular weight[a]		M_w/M_n[b]	DE[c]	
		M_w (DP)	M_n (DP)		Chemical method	HPSEC Method (M_n)
LC	15	30,270 (186.9)	4,052 (25.0)	7.5	4.3±0.1	4.0±0.2
	30	8,729 (53.9)	2,127 (13.1)	4.1	8.3±0.3	7.6±0.4
	60	3,771 (23.3)	1,217 (7.5)	3.0	14.7±0.3	13.3 ± 0.2
SC	15	27,868 (172.0)	5, 134 (31.7)	5.4	4.5±0.1	3.2±0.1
	30	10,451 (64.5)	2,438 (15.1)	4.3	7.9±0.2	6.6±0.1
	60	6,015 (37.1)	1,906 (8.5)	3.2	12.2±0.3	11.8±0.3
D45	15	2,947 (18.2)	2,280 (14.1)	1.3	6.5±0.1	7.1±0.3
	30	2,264 (14.0)	1,481 (10.9)	1.5	8.2±0.1	9.1±0.1
	60	1,744 (10.8)	1,246 (7.7)	1.4	11.9±0.4	13.0±0.1

Values are the average of triplicate determinations.
[a] Average molecular weight by weight, M_w, and by number, M_n, and (degree of polymerization).
[b] Polydispersity.
[c] Dextrose equivalence.

and D45 hydrolysates obtained by using chemical and HPSEC (M_n) methods are shown in Table 1. The polydispersity of the D45 hydrolysates was small (1.3–1.5) and remained relatively steady throughout hydrolysis, indicating that D45 hydrolysates had great uniformity, irrespective of the degree of starch hydrolysis. The polydispersity of the LC and SC hydrolysates was much larger than for the D45 hydrolysates because the LC and SC hydrolysates exhibited bimodal product distributions, covering wider ranges of molecular weight, whereas the D45 hydrolysates displayed unimodal distribution covering a smaller range of molecular weight (Fig. 1(A)–(C)). The DE values for the enzyme hydrolysates, measured by the HPSEC number-average method, were in reasonable agreement with those obtained by the chemical method (Table 1). The DE values for the LC and SC hydrolysates (bimodal distribution) obtained from chemical methods were slightly larger than the DE values from the HPSEC number-average method, whereas the DE values for D45 hydrolysates (mono-distribution) showed the reverse (Table 1).

Oligosaccharides (DP up to 13) present in the α-amylase hydrolysates separated by TLC are shown in Fig. 2. Normalized concentrations obtained by measuring the color intensity of products on TLC plates (Fig. 2) are presented in Fig. 3(A)–(C). During the early stages of hydrolysis, (DE 4.3), LC produced mainly G5, G6, G7, G8, G12 and G13 (Fig. 3(A)) in addition to larger products that remained at the origin. At DE 4.5, SC produced mainly G6, G7, G8, G9 and G10 (Fig. 3(B)) in addition to larger products, whereas D45, at DE 6.5, produced mainly G7, G8, G9 and G10 (Fig. 3(C)) and larger products. As hydrolysis progressed, G2, G3, G5, G6 and G7 became the main products of LC at DE 14.7; G4, G5, G6 and G7 were the main products for SC at DE 12.2, and G6, G7, G8 and G9 for D45 at DE 11.9. G5 was a minor product of D45 at all tested DE values (Fig. 3(C)). Overall, the results indicated that in addition to very large products with DP 152 and 113, LC and SC produced oligosaccharides that were smaller than those produced by D45. Moreover, some oligosaccharides larger than G7 in the α-amylase

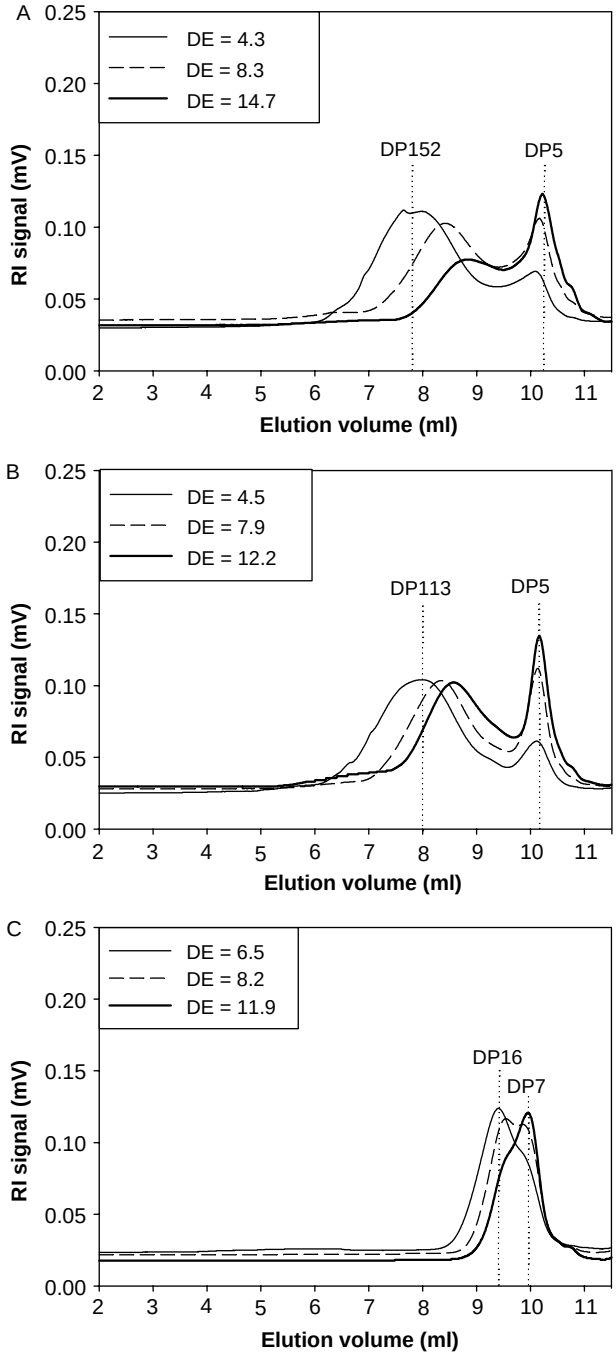

Fig. 1. HPSEC chromatograms of LC (A), SC (B) and D45 (C) hydrolysates of waxy maize starch at different DE values.

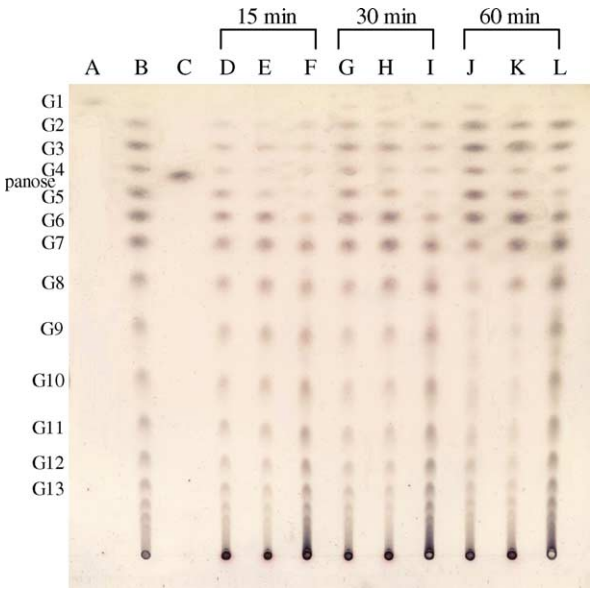

Fig. 2. TLC chromatograms (4 ascents) of LC, SC and D45 hydrolysates of waxy maize starch at different DE values and reaction time (15, 30, and 60 min): D-glucose (lane A), linear maltodextrins (lane B), panose (lane C), and LC (lane D [DE 4.3], G [DE 8.3] and J [DE 14.7]), SC (lane E [DE 4.5], H [DE 7.9] and K [DE12.2]) and D45 (lane F [DE 6.5], I [DE 8.2] and L [DE11.9]).

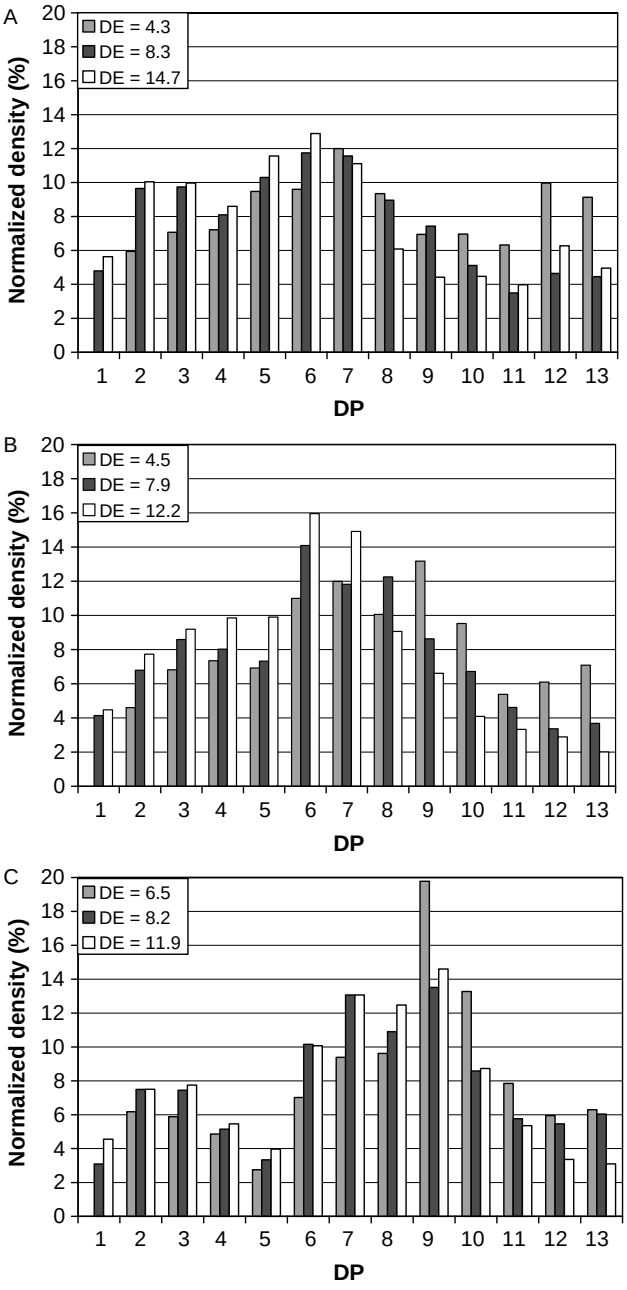

Fig. 3. Comparison of normalized iodine stain-intensity distributions of LC (A), SC (B) and D45 (C) hydrolysates of waxy maize starch at different DE values separated by TLC as shown in Fig. 2.

hydrolysates were eluted between linear oligosaccharides; these were less concentrated in the LC and SC than in D45 hydrolysates (Fig. 2). Based on the observation that their R_f values did not match those of the linear maltodextrin standards, these products were likely branched oligosaccharides. The larger concentration of branched oligosaccharides found in the D45 hydrolysate could be attributed to more oligosaccharides being produced by D45 than the other two enzymes. No panose was detected in any of the amylase hydrolysates within the reaction times studied (Fig. 2).

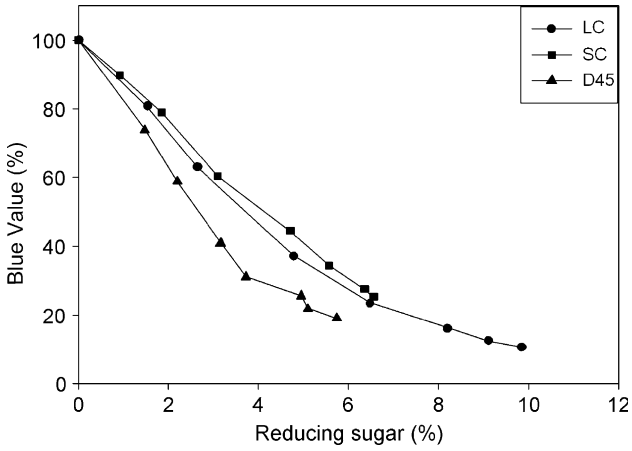

Fig. 4. Comparison of the decrease in blue value (%) with the increase in reducing sugar (%) of the enzyme hydrolysis of amylose by LC, SC and D45.

Plots of iodine-stained blue value against reducing sugar content of amylose hydrolyzed by LC, SC and D45 are shown in Fig. 4. For an equivalent decrease in blue value, the SC hydrolysate displayed a slightly higher concentration of reducing sugar than did the LC hydrolysate, whereas D45 hydrolysate contained a substantially lower concentration of reducing sugar. These results suggest that SC and LC have more multiple-attack reaction (Robyt and French, 1967), whereby the enzyme forms a complex with an amylose chain, and catalyzes the hydrolysis of several consecutive bonds before it dissociates. α-Amylases that display a multiple-attack reaction pattern typically produce more small reducing-sugars, without significantly reducing the length of the amylose chain. Thus a hydrolysate resulting from such an enzyme reaction exhibits darker blue color when stained with iodine (Robyt and French, 1967). In contrast, D45 displayed more random attack (also termed a multichain action pattern Robyt and French, 1967) and quickly reduced the chain length of the amylose. This conclusion was supported by the molecular weight distribution profiles of waxy maize starch hydrolyzed by LC, SC and D45 (Fig. 1(A)–(C)). D45 attacked the amylopectin substrate randomly resulting in a rapid decrease in amylopectin molecular weight and a mono-distribution of products (Fig. 1(C)). Small branched-oligosaccharides were produced primarily by this multichain action pattern, as seen in the TLC analysis (Fig. 2). On the other hand, the multiple attack action of LC and SC resulted in hydrolysis of several amylopectin linkages toward the non-reducing end of the chain before dissociation occurred (Robyt & French, 1970). Thus, the molecular weight distribution of products exhibited a bimodal distribution with highly branched amylopectin fragments, and small molecular-weight oligosaccharides resulting from the multiple attacks (Fig. 1(A) and (B)). The proposed reaction pattern schematic diagrams of LC, SC and D45 are shown in Fig. 5.

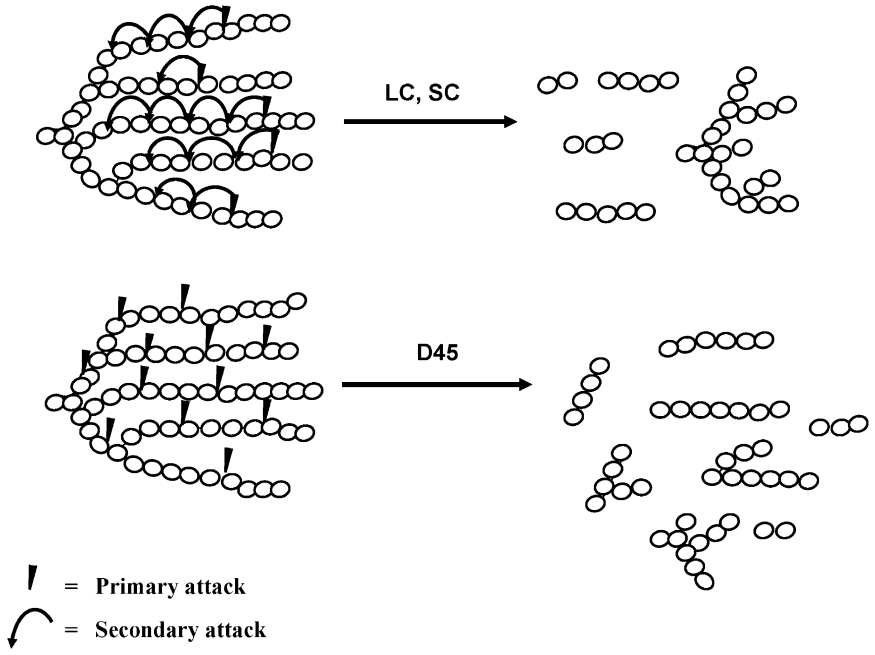

Fig. 5. Proposed reaction patterns schematic diagrams of LC, SC and D45 amylopectin hydrolysis. LC and SC have multiple-attack reaction patterns whereas D45 has a random-attack reaction pattern.

3.2. Structure of α-limit dextrins

Fig. 6 shows HPSEC profiles for the α-limit dextrins produced by the action of LC, SC and D45 on waxy maize starch. The α-limit dextrins produced by D45 displayed one peak at DP 8 and another peak at DP 2, whereas those produced by LC and SC showed primarily one peak centered at DP 5 with a shoulder at DP 3. Average DP's of these limit dextrins are shown in Table 2. The D45 α-limit dextrins had a slightly longer average DP than those produced by LC and SC (Table 2).

Proteinase K was used to inactivate the α-amylases before analysis of the branched structures of the α-limit dextrins. The α-limit dextrins produced by LC, SC and D45 were debranched with pullulanase. The average DP's of the debranched products are shown in Table 2 and are slightly less than the non-debranched counterparts (Table 2).

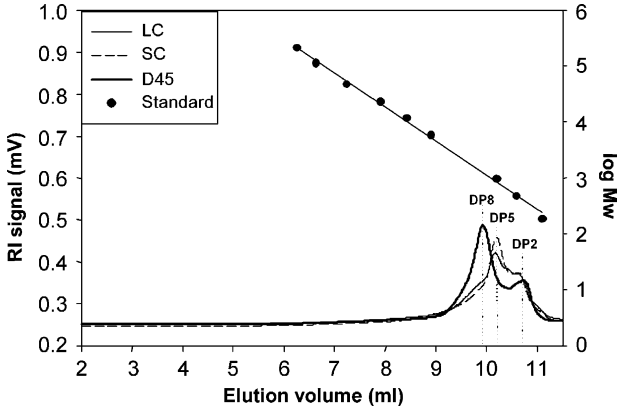

Fig. 6. HPSEC chromatograms of LC, SC and D45 α-limit dextrins from waxy maize starch.

The fine structure of the debranched α-limit dextrins was analyzed by using TLC and was compared with the structure of the non-debranched limit dextrin as shown in Fig. 7. Spots that did not match R_f values of linear maltodextrins were designated as branched oligosaccharides (B1–B6). TLC results showed that the largest linear oligosaccharide was DP 5 in the LC α-limit dextrin, DP 6 for the SC α-limit dextrin and DP 8 for the D45 α-limit dextrin. Linear oligosaccharide with DP6 has been reported as a major product of amylase hydrolysis by *B. subtilis* α-amylase (Robyt & French, 1963). No panose was found before or after debranching in any samples tested (Fig. 7). The TLC results showed that branched α-limit dextrins produced by the three α-amylase species were debranched by pullulanase.

4. Conclusion

Results of this study suggest that amylase D45 is a liquefying endoamylase capable of cleaving α-1, 4-glycosidic bonds present in the inner part of amylopectin producing various linear and branched oligosaccharides. The reaction pattern of D45 showed more random attack (multichain), whereas that of amylases LC and SC displayed more multiple-attack and

Table 2
Degree of polymerization (DP) of LC, SC and D45 α-limit dextrins before and after debranching with pullulanase

α-Limit dextrins	Average DP[a]	
	Before debranching	After debranching
LC	3.6±0.0	3.4±0.1
SC	4.6±0.2	4.0±0.1
D45	4.8±0.1	4.4±0.2

[a] Values are the average of triplicate determinations±standard deviation.

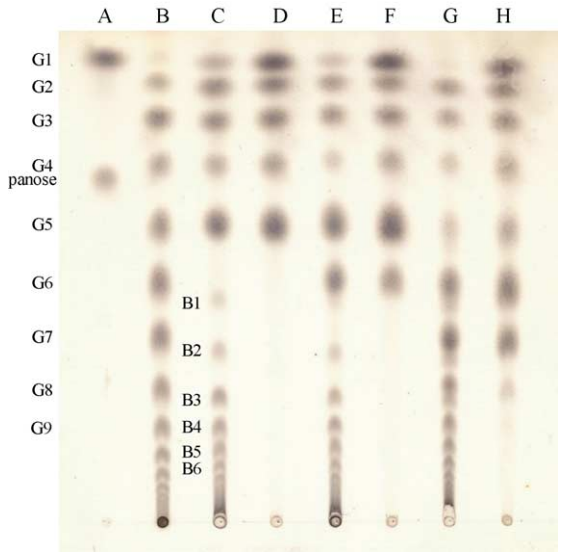

Fig. 7. TLC chromatograms (2 ascents) of α-limit dextrins with and without debranching: D-glucose and panose (lane A), linear maltodextrins (lane B), LC α-limit dextrins (lane C), debranched LC α-limit dextrins (lane D), SC α-limit dextrins (lane E), debranched SC α-limit dextrins (lane F), D45 α-limit dextrins (lane G), and debranched D45 α-limit dextrins (lane H).

resulted in production of small reducing-sugars from cleavage of consecutive glycosidic linkages. The results indicate that LC and SC are able to hydrolyze α-1, 4-glycosidic bonds of amylopectin molecules closer to the branch linkage to produce α-limit dextrins with shorter branch chains than D45.

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