Articles

Temperature Impacts the Multiple Attack Action of Amylases

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The action pattern of several amylases was studied at 35, 50, and 70 °C using potato amylose, a soluble (Red Starch) and insoluble (cross-linked amylose) chromophoric substrate. With potato amylose as substrate, *Bacillus stearothermophilus* α-amylase (BStA) and porcine pancreatic α-amylase displayed a high degree of multiple attack (DMA, i.e., the number of bonds broken during the lifetime of an enzyme−substrate complex minus one), the fungal α-amylase from *Aspergillus oryzae* a low DMA, and the α-amylases from *B. licheniformis*, *Thermoactinomyces vulgaris*, *B. amyloliquifaciens*, and *B. subtilis* an intermediate DMA. These data are discussed in relation to structural properties of the enzymes. The level of multiple attack (LMA), based on the relation between the drop in iodine binding of amylose and the increase in total reducing value, proved to be a good alternative for DMA measurements. The LMA of the endo-amylases increased with temperature to a degree depending on the amylase. In contrast, BStA showed a decreased LMA when temperature was raised. Furthermore, different enzymes had different activities on Red Starch and cross-linked amylose. Hence, next to the temperature, the action pattern of α-amylases is influenced by structural parameters of the starch substrate.

Introduction

The unique physical properties and behavior of starch make it an important functional ingredient in many products. To meet industrial demands, modification of starch is often needed.[1−4] In this respect, the amylase-catalyzed hydrolysis of starch is among the most important industrially applied enzyme reactions.[5]

Endo-amylases [α-amylase (EC 3.2.1.1), glycoside hydrolase (GH) family 13] hydrolyze internally the α-(1,4) bonds of the starch components, the essentially linear amylose, and the highly branched amylopectin. Exo-amylases [such as β-amylase (EC 3.2.1.2), GH family 14] degrade the starch molecules successively from the non-reducing end. That amylases from various sources degrade starch in a different, nonrandom manner has first been discussed decades ago.[6−8] The differences in action pattern have been explained on the basis of processive attack. In this view, an amylase cleaves several glycosidic bonds after the first random hydrolytic attack and then dissociates from the substrate.[9] In the case of an amylase, the processive attack is often referred to as multiple attack. The multiple attack mechanism has first been described for β-amylase,[4,10] and subsequently for α-amylases.[8,11−14] The multiple attack mechanism is an intermediate between the "single-chain" and the "multi-chain" mechanism. In the former, the polymer molecule is hydrolyzed completely before dissociation of the enzyme−substrate complex, while, in the latter, only one bond is hydrolyzed per effective encounter. Robyt and French (1967)[8] defined the degree of multiple attack (DMA) as the number of bonds broken during the lifetime of an enzyme−substrate complex minus one (i.e., the initial random cleavage).

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During the past decades, most research on multiple attack was performed on the (porcine) pancreatic α-amylase (PPA). Several methods have been developed to evaluate the multiple attack action of α-amylases.[7,8,12] They all show a high DMA for PPA, but, for the few other α-amylases tested, they gave divergent results.

Concerning the temperature effect on DMA, results were found to be inconsistent. Mazur and Nakatani (1993)[14] concluded that the DMA of PPA increases with temperature. Furthermore, Marchal and co-workers (1999)[15] reported that, for the same level of bonds hydrolyzed, the saccharide composition depends on the hydrolysis temperature for α-amylases from *Bacillus licheniformis*, *B. amyloliquefaciens*, and *B. stearothermophilus*. While it seems plausible that a temperature effect on DMA would explain these results, Kramhøft and Svensson (1998)[16] found that, for a *B. licheniformis* α-amylase, DMA did not change with temperature.

Furthermore, the DMA of PPA depends on pH. Robyt and French (1967)[8] found for PPA a decrease of DMA from 6.0 to 1.7 when the pH was raised from pH 6.9 to 10.5. The decrease in DMA was explained on the basis of a probable change to an unfavorable ionization state of one or more catalytic groups, thereby retarding them to an extent where they rarely would function more than once during an effective encounter. In contrast, when the pH was decreased to pH 4.7, DMA did not change. Kramhøft and co-workers (2005)[17] found also for PPA a DMA of 5.9 at pH 5.5. Marchal and co-workers (1999)[15] found that varying the pH in a 5.1−7.6 interval at 70 °C did not significantly influence the saccharide composition obtained during *B. licheniformis* α-amylase hydrolysis.

Most of the experimental research on the processivity of amylases has been done using the linear amylose substrate. However, in general, amylose presents only a relatively small

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fraction of starch. The main fraction consists of branched amylopectin, which evidently is the main substrate in starch hydrolysis. On the basis of their experimental results, Mazur and Nakatani (1993)[14] suggested that an amylase can act on the side chains of the branched amylopectin with a multiple attack mechanism. Nevertheless, the multiple attack action can be expected to be restricted by the branched structure. Hence, the nature of the substrate probably determines the number of repeated attacks (or the DMA-value) on nonlinear, branched substrates such as amylopectin.

The physicochemical behavior of starch defines its functionality in applications and can be related to its complex structure. How the structure of starch is altered upon amylase-catalyzed hydrolysis depends on its action pattern. In this respect, the mode of action of an amylase, in particular its DMA-value, is an important feature when enzymic modification of starch is considered. Because of the limited information on the action pattern of amylases other than PPA, we studied the action pattern of several amylases at three different temperatures (35, 50, and 70 °C) using three different substrates (potato amylose and two chromophoric substrates). This way, the action pattern of amylases was characterized, and the effect of temperature and the influence of substrate on the amylase action pattern were evaluated.

Experimental Section

Materials. Amylose (DP ≈ 220) from potato starch was a gift of Tate & Lyle (Aalst, Belgium). Amylazyme tablets and Red Starch were purchased from Megazyme (Bray, Ireland). Soluble starch was obtained from Merck (Darmstadt, Germany). Endo-α-amylases from *Bacillus subtilis* (BSuA), *B. amyloliquefaciens* (BAA), as well as PPA were purchased from Sigma Aldrich Chemie (Bornem, Belgium). Endo-α-amylase from *B. licheniformis* (BLA), fungal endo-α-amylase from *Aspergillus oryzae* (TAKA), and *β*-amylase from *B. cereus* (BCB) were obtained from Megazyme (Bray, Ireland). Endo-α-amylase type I from *Thermoactinomyces vulgaris* R-47 (TVA)[18,19] was kindly donated by Prof. Sakano (University of Agriculture and Technology, Department of Applied Biological Science, Tokyo, Japan). Maltogenic α-amylase from *B. stearothermophilus* (Novamyl) (BStA) was obtained from Novozymes (Bagsvaerd, Denmark). All enzymes were free of any interfering starch degrading or modifying activity as checked by size exclusion chromatography and activity measurements.

Amylase Activity Assay. Amylase activity was assayed using soluble starch (1.0%) as substrate in 100 mM sodium maleate buffer (pH 6.0) containing 5.0 mM calcium chloride at 35, 50, and 70 °C. Substrate solution and suitably diluted enzyme preparations were preincubated at the respective temperatures for 5 min. Next, substrate solution (500 μL) was added to enzyme preparation (200 μL). After 10 min at the respective temperatures, the reaction was stopped with an alkaline copper solution, and the reducing end groups liberated were quantified (Somogyi, 1952)[20] using maltose (0−0.7 μmol) as a standard. Results are expressed as μmol of maltose equivalents released per minute at the respective temperature.

Multiple Attack Assay. Multiple attack was studied by a method based on Robyt and French (1967)[8] and actualized by Kramhøft et al. (2005),[17] with some modifications. Amylose (100 mg) was dissolved in 2.0 mL of hot 90% dimethyl sulfoxide and diluted with 80 mL of water (100 °C). The solution was kept at 100 °C for 30 min, and then cooled to room temperature. Sodium maleate buffer (10.0 mL, 250 mM, pH 6.0) containing 50 mM $CaCl_2$ and 2.5 mg/mL bovine serum albumin was added, and the volume was adjusted to 100 mL with water. Based on the optimal pH of the different enzymes and the conditions for DMA analysis found in literature, pH 6.0 was chosen, which for most of the enzymes is close to or somewhat lower than the optimal pH. DMA will hence be minimally influenced by the pH.[8,15,17]

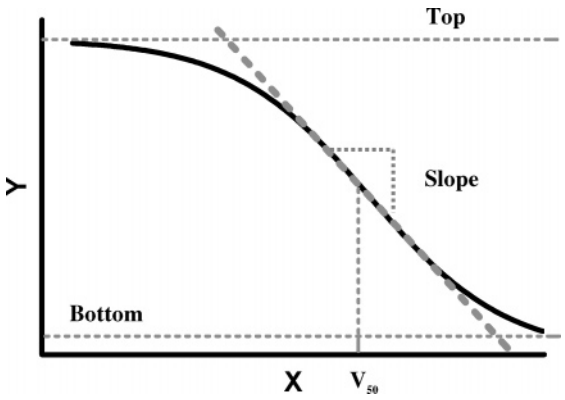

Figure 1. A typical curve described by the Boltzmann sigmoidal equation: $y = Bottom + [\{(Top - Bottom)\}/\{1 + \exp(\{V_{50} - x\}/Slope)\}]$.

The amylose solution (24.5 mL) was equilibrated at 35, 50, or 70 °C, and 0.5 mL of an enzyme solution, releasing 0.5 μmol of maltose per milliliter from soluble starch per minute at the same temperature and pH 6.0, was added to initiate the reaction. During 120 min, aliquots (1.5 mL) were withdrawn at 10 min time intervals and acidified by addition of 750 μL of HCl (1.0 M) to stop the reaction.

To determine the blue value of amylose−iodine complex of the hydrolysate, 750 μL of the above acidified solution was mixed with 250 μL of iodine reagent (0.2% KI and 0.02% I_2) and diluted with 4.0 mL of water. Next, the absorbance was measured at 620 nm and expressed as a percentage of the absorbance of the aliquot at time point zero [further referred to as the (relative) blue value].

The remaining acidified solution was neutralized with 500 μL of 1.0 M NaOH. First, 700 μL of this neutralized solution was used to determine the reducing sugar concentration (further referred to as total reducing value), with the Somogyi−Nelson method,[20] with maltose (0−0.7 μmol) as standard. Second, the polysaccharides in 600 μL of the neutralized solution were precipitated by adding 99.9% ethanol (1.2 mL). After 48 h at 4 °C, the mixtures were centrifuged (16 000*g*, 5 min), washed three times with 1.2 mL of ethanol (70%), and air-dried. The precipitate was redissolved in 60 μL of 1.0 M KOH, diluted with 600 μL of water, and neutralized with 60 μL of 1.0 M HCl. The reducing value of the polysaccharide fraction was determined by the copper-bicinchoninate-method described by Waffenschmidt and Jaenicke (1987),[21] with maltose (0−0.027 μmol) as standard. To mimic samples, 20 μL of 1.0 M KOH and 20 μL of 1.0 M HCl were added to the maltose standards. Each analysis was performed at least three times with duplicate measurements.

For each time point, the relative blue value (*y* in Figure 1) was plotted versus the respective total reducing value (*x* in Figure 1). The experimental results were analyzed using the software GraphPad Prism version 3.03. Nonlinear regression was performed using the Boltzmann sigmoidal equation:

$$y = Bottom + \frac{Top - Bottom}{1 + \exp\left(\frac{V_{50} - x}{Slope}\right)} \quad (1)$$

The parameters *Top*, *Bottom*, V_{50} (the *x*-value at which the *y*-value is halfway between Bottom and Top), and *Slope* (Figure 1) were estimated by the software. RV_{50} [=reducing value (*x*) corresponding to a decrease in relative blue value (*y*) to 50%] and RV_{80} [=reducing value (*x*) corresponding to a decrease in relative blue value (*y*) to 80%] were calculated from eq 1 with *y* equaling 50 and 80, respectively.

Finally, for the time points with a relative blue value of at least 80%,[17] the total reducing value was plotted against the respective reducing value of the polysaccharide fraction, and a linear regression line was fitted. The degree of multiple attack (DMA) was calculated as the slope of this regression line minus 1.

Table 1. Overview of Parameters Characterizing the Action Pattern of Amylases at 35 °C and pH 6.0, Determined Using 0.1% Potato Amylose as a Substrate[a]

	DMA	V_{50}	*Slope*	RV_{50}	RV_{80}
TAKA	2.5 ± 0.2 e	0.32 ± 0.04	−0.090 ± 0.023	0.32 ± 0.03	0.21 ± 0.01
BSuA	3.1 ± 0.5 de	0.34 ± 0.06	−0.067 ± 0.010	0.37 ± 0.06	0.27 ± 0.05
BAA	3.9 ± 0.4 c	0.35 ± 0.00	−0.087 ± 0.008	0.37 ± 0.01	0.26 ± 0.00
BLA	4.7 ± 0.6 c	0.43 ± 0.01	−0.109 ± 0.005	0.46 ± 0.01	0.32 ± 0.01
TVA	3.8 ± 0.2 cd	0.46 ± 0.01	−0.093 ± 0.034	0.45 ± 0.05	0.31 ± 0.04
PPA	8.0 ± 0.7 b	0.92 ± 0.19	−0.175 ± 0.042	0.93 ± 0.19	0.70 ± 0.14
BStA	12.1 ± 2.1 a	1.67 ± 0.20	−0.260 ± 0.017	1.71 ± 0.07	1.29 ± 0.11

[a] DMA, degree of multiple attack; V_{50} and *Slope*, parameters from the Boltzmann sigmoidal regression estimated by the software GraphPad Prism; RV_{50} and RV_{80}, parameters calculated from the Boltzmann sigmoidal regression equation; TAKA, *Aspergillus oryzae* α-amylase; BSuA, *Bacillus subtilis* α-amylase; BAA, *B. amyloliquefaciens* α-amylase; BLA, *B. liceniformis* α-amylase; TVA, *Thermoactinomyces vulgaris* α-amylase I; PPA, porcine pancreatic α-amylase; and BStA, *B. stearothermophilus* maltogenic α-amylase. Values are means with corresponding standard deviations of at least three individual experiments. Different letters indicate significant different DMA-values as determined by a *t*-test with $P < 0.05$.

Statistical Evaluation. Results were evaluated statistically by the *t*-test (PROC ANOVA). Results are considered significantly different for $P < 0.05$. Statistical analysis was performed using the Statistical Analysis System software (v. 8.1, SAS Institute, Cary, NC).

Action Pattern with Chromophoric Substrates. *Azurine-Cross-linked Amylose.* Aliquots (1.0 mL) of a series of enzyme dilutions were pre-equilibrated at 35, 50, or 70 °C for 5 min. Azurine-cross-linked amylose, in an Amylazyme tablet form, was added to each aliquot (without stirring) and incubated at appropriate temperatures. After exactly 10 min, 10.0 mL of a Trizma base solution (2.0%, w/v) was added, and the reaction mixture was vigorously stirred to terminate the reaction. After 5 min at room temperature, the reaction mixtures were stirred again and filtered through filter paper (Ø 90 mm). The absorbance of the filtrate was measured at 590 nm against a reaction blank and plotted against the amylase activity (expressed as μmol of maltose released per minute from soluble starch at the appropriate temperature[20]) of the series of enzyme dilutions. Each analysis was performed at least three times with triplicate measurements.

Soluble Red Starch. A series of enzyme dilutions and Red Starch (2.0%, w/v) solutions were separately preincubated at 35, 50, or 70 °C for 5 min. An aliquot (1.0 mL) of the enzyme dilution was added to 0.5 mL of the Red Starch solution and incubated at the appropriate temperature for exactly 10 min. The reaction was terminated by adding 2.5 mL of ethanol (95% v/v) with vigorous stirring. The reaction tubes were allowed to equilibrate to room temperature for 10 min and then centrifuged at 1000*g* for 10 min. The supernatant solution was measured at 510 nm against a reaction blank. The absorbance of the supernatant was plotted against the reducing value (expressed as μmol of maltose released per minute from soluble starch at the appropriate temperature[20]) of the series of enzyme dilutions. Each analysis was performed at least three times with triplicate measurements.

For both substrates, a linear regression line was fitted to the experimental data, using the method of least-squares.

Results and Discussion

Action Pattern on Potato Amylose in Solution at 35 °C. For every amylase, the action pattern, reflecting whether an amylase is able to hydrolyze more than one bond after the first random hydrolytic attack and before dissociating from the substrate, was deduced from the relation between the drop in relative blue value and the increase in the total reducing value during amylolysis.[8] In addition, DMA-values were determined for every amylase (Table 1).

Figure 2 plots the relative blue value versus the total reducing value for eight different amylases at 35 °C and pH 6.0. This figure shows that, for equal levels of reducing sugars generated, which indicates an equal total number of hydrolytic attacks, the various α-amylases induce a different drop in relative blue value, which is correlated to the corresponding decrease in molecular weight of amylose. Therefore, the amylases display various levels of multiple attack (LMA) and cannot be considered as hydrolyzing starch in a truly random manner. At a given level of reducing sugars released, PPA resulted in a smaller decrease in blue value than the other endo-α-amylases. This was reflected in a high DMA-value (8.0 ± 0.7), which was significantly different from that of the other endo-α-amylases. Earlier investigations showed DMA-values for PPA of 6.0 ± 0.9 at 40 °C and pH 6.9[8] and 5.9 ± 1.6 at 37 °C and pH 5.5.[20] In contrast, TAKA resulted in a rapid decrease of the relative blue value with increasing reducing value, in accordance with a low DMA (DMA = 2.5 ± 0.2). A similar DMA-value (1.9 ± 0.4 at 40 °C and pH 5.5) has been reported earlier.[8] The curves of the other α-amylases, except of the maltogenic BStA, are situated between those of PPA and TAKA. Based on the DMA-values, the α-amylases, except PPA and BStA, can be divided into two groups. The first group consists of BLA (DMA = 4.7 ± 0.6), TVA (DMA = 3.8 ± 0.2), and BAA (DMA = 3.9 ± 0.4) and the second of BSuA (DMA = 3.1 ± 0.5) and TAKA (DMA = 2.5 ± 0.2). The DMA values of BLA and BAA are significantly different from those of BSuA and TAKA, while that of TVA is only significantly different from that of TAKA. For an α-amy-

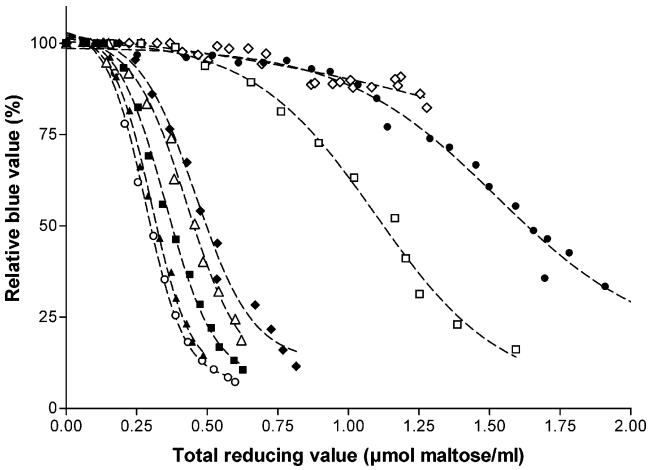

Figure 2. Relation between the drop in relative blue value (as percentage of the absorbance of the aliquot at time point zero) and the increase in total reducing value during amylolysis at 35 °C and pH 6.0, using 0.1% potato amylose as a substrate. (○) TAKA, *Aspergillus oryzae* α-amylase; (▲) BSuA, *Bacillus subtilis* α-amylase; (■) BAA, *B. amyloliquefaciens* α-amylase; (△) BLA, *B. licheniformis* α-amylase; (◆) TVA, *Thermoactinomyces vulgaris* α-amylase I; (□) PPA, porcine pancreatic α-amylase; (●) BStA, *B. stearothermophilus* maltogenic α-amylase; (◇) BCB, *B. cereus* β-amylase. The dashed lines shown are the Boltzman sigmoidal lines fitted to the experimental points.

lase from *B. licheniformis*, Kramhøft and Svensson (1998)[16] reported a DMA-value of 1.2 (37 °C, pH 5.5), a value considerably lower than that reported here for BLA. However, the enzyme used by these authors may differ from that used in the current experimental work. In general, the relative order of the curves was reflected in the DMA-values.

For the maltogenic amylase BStA, the drop in blue value was negligible until a considerable level of reducing sugars (>2 μmol/mL) was generated. This corresponds to earlier findings, in which, in Rapid Visco Analysis studies, BStA was found to affect the amylose population in a specific manner, while largely maintaining its molecular weight.[22,23] These observations suggest that its action pattern is comparable to that of the exo-acting enzyme BCB. However, this contrasts with the findings that BStA does not require a non-reducing end, attacks amylose in an endo-like manner, and acts with net retention of the anomeric configuration.[24,25] An alternative explanation is that BStA is an endo-acting amylase with a high DMA, a suggestion already made by Dauter and co-workers (1999),[25] and which is in line with its DMA-value of 12.1 ± 2.0.

The curve of the exo-amylase BCB showed hardly any change in blue value with increasing reducing value, in agreement with the fact that the β-amylase liberates maltose from the non-reducing end, resulting in poor reduction of the amylose molecular weight.

The molecular basis of the multiple attack action mechanism is not well understood. Several authors have commented on the structural requirements of the enzyme for its multiple attack action.[8,9,20,26] Although the study of the multiple attack action mechanism was beyond the scope of the present work, our data on multiple α-amylases, representing a range of DMA-values, could substantiate some of the suggestions and speculations made in literature.

According to Breyer and Matthews (2001),[9] processivity or the multiple attack action of enzymes is related to the degree of enclosure of the substrate by the enzyme. In general, cleft-shaped binding sites are found in endo-acting amylases, whereas exo-acting amylases have a pocket-shaped active site.[27] Although both structures allow a partial enclosure of the substrates, not all endo-amylases have a high DMA. Hence, this shows that the general shape of the active site is not the only prerequisite for multiple attack. In this respect, Breyer and Matthews (2001)[9] proposed that processivity can be achieved by the enzyme having a large interaction surface with the substrate.

A first aspect of the interaction between enzyme and substrate can be related to the active site of the enzyme. Indeed, the active site is composed of an array of subsites, each of which interacts with individual sugar residues of the substrate. Kramhoft and Svensson (1998)[16] proposed a possible relation between the number of subsites and DMA. They determined a high DMA for PPA having five subsites and a low DMA for amylases with 9 to 10 subsites. In this view, and somewhat in contrast to the suggestion by Breyer and Matthews (2001),[9] amylases with a shorter substrate binding cleft (lower number of subsites) have a higher DMA. When DMA-values in the present work were related to the respective number of subsites, our results were found to be in line with this theory with BStA[24,25] and PPA[28] (high DMA) having five subsites, BLA[29] and TVA[19] (intermediate DMA) having eight subsites, and BSuA[30] and TAKA[31] (low DMA) having nine subsites. Furthermore, Kramhøft and co-workers (2005)[20] clearly showed the importance of the subsites in determining DMA. Using barley α-amylase AMY-I mutants, they observed that structural changes in the substrate binding cleft alter the DMA of this enzyme. Particularly, a single mutation at subsite −6 led to a looser substrate binding at this subsite and resulted in an increased DMA.[17] However, other mutations could counteract this increase, or drastically lower the DMA. These authors suggested that changes in the substrate binding cleft can result in a less efficient repositioning of substrate without dissociation (substrate sliding), probably due to difficulty in accommodating the stable left-hand helical substrate conformation.

A second aspect of the interaction between enzyme and substrate can be related to the presence of carbohydrate binding regions outside the active site. In this respect, the fusion of AMY-1 with a starch binding domain (SBD) resulted in an increased DMA.[17] Furthermore, putative starch interaction sites outside the active site have been identified for BStA (a starch-binding domain E)[25] and PPA (two independent carbohydrate recognition sites),[32] which have been suggested to be involved in the multiple attack action. Because BStA and PPA were the α-amylases with the highest DMA-values in the present work, this seems to further substantiate the importance of such starch interaction sites in determining DMA. In addition, for BLA, BAA, and BSuA with intermediate to low DMA, no indication of the presence of additional binding sites was found in literature. In contrast, although TVA possesses a starch-binding domain (domain N),[19] the enzyme has only an intermediate DMA. Likewise, Vujicic-Zagar and Dijkstra (2006)[33] recently reported for an *A. niger* α-amylase (with 100% sequence identity to TAKA) the presence of additional carbohydrate binding sites outside the active site, which could function to bind the polysaccharide chain extending from the active site, but the TAKA enzyme has the lowest DMA. This would indicate that the low DMA of TAKA and TVA could presumably be attributed to the large number of subsites or the architecture of their active site.

From the above, we believe a picture emerges in which the multiple attack action of an amylase can be related to the size of the interaction surface between enzyme and substrate (as positively affected by the presence of substrate binding domains or carbohydrate recognition sites outside the active site) and to the degree to which the active site architecture and properties (as influenced by the number of subsites or the specific amino acids in those subsites) allow the reorganization (sliding) of the substrate with its own specific helical conformation.

RV_{80}-Value as an Expression for LMA. Given the laborious character of the procedure to determine amylase DMA, we tried to relate DMA to parameters derived from the curves describing the drop in relative blue value against the increase in amount of reducing sugars (Figure 2). For each amylase, four different parameters were obtained, that is, V_{50} and *Slope*, which were estimated by the software when fitting the curve to the data, and RV_{50}- and RV_{80}-values calculated from eq 1. In general, a good correlation existed between DMA and the different parameters, with the correlation between DMA and RV_{50} ($R^2 = 0.98$), and between DMA and RV_{80} ($R^2 = 0.98$) being the best. Thus, both RV_{80} and RV_{50} are good approximations for evaluation of DMA of the different enzymes.

For the determination of DMA, the experiment has to be performed during the initial stage of hydrolysis to be able to exclude bias from secondary hydrolysis of products. Kramhøft and co-workers (2005)[17] defined the initial state of amylose hydrolysis as the interval characterized by constant (RV_t/RV_p) with RV_t, the rate of reducing sugar formation in the total digest and RV_p the rate of reducing sugar formation in the polysaccharide fraction. In the present work, only samples whose blue

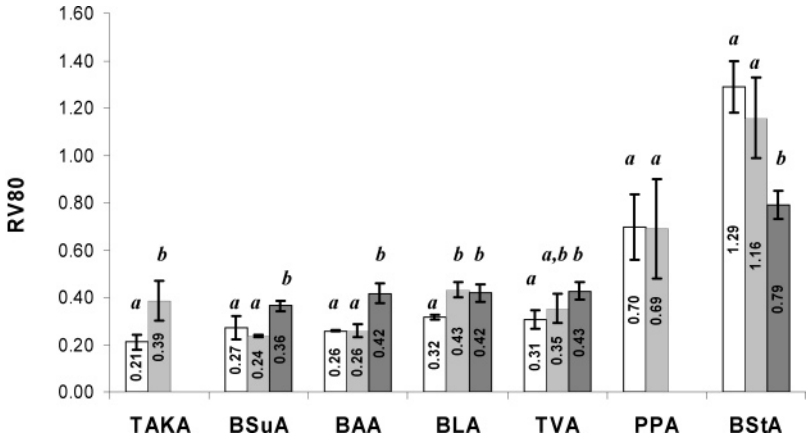

Figure 3. Overview of RV_{80} at 35 °C (□), 50 °C (light grey), and 70 °C (dark grey) and pH 6.0 using 0.1% potato amylose as a substrate. RV_{80}: parameter calculated from the Boltzmann sigmoidal regression equation. Values are means with corresponding standard deviations of at least three individual experiments. *a*,*b*, for each enzyme, different letters indicate significantly different RV_{80}-values at different temperatures as determined by a *t*-test with $P < 0.05$; TAKA, *Aspergillus oryzae* α-amylase; BSuA, *Bacillus subtilis* α-amylase; BAA, *B. amyloliquefaciens* α-amylase; BLA, *B. licheniformis* α-amylase; TVA, *Thermoactinomyces vulgaris* α-amylase I; PPA, porcine pancreatic α-amylase; and BStA, *B. stearothermophilus* maltogenic α-amylase.

value had not dropped below 80% were used to calculate DMA because their corresponding total reducing value and reducing value of the polysaccharides increased linearly by time, meaning they were within the initial stage of hydrolysis.

Moreover, because the procedure to determine RV_{80} (plotting the relative blue value versus the total reducing value) is part of the procedure to determine DMA (plotting reducing value of the polysaccharide fraction versus the total reducing value), the high correlation between DMA and RV_{80} is to be expected. Furthermore, a low reducing value of the polysaccharide fraction for a certain total reducing value reflects a rather high molecular weight of this fraction. Because the relative blue value of the digest is related to the chain length of the substrate and consequently to the molecular weight of amylose,[34] a low reducing value of the polysaccharide fraction for a certain total reducing value implies a high relative blue value. Hence, both LMA and DMA relate the molecular weight of the polysaccharide fraction to the total reducing value. We are convinced that this is true independent of the temperature used. Furthermore, based on preliminary DMA-values at 50 °C, the correlation between DMA and RV_{80}-values at 50 °C was similar to that at 35 °C (results not shown).

Hence, the use of RV_{80} (Figure 3) as an alternative for DMA is justified for studying the effect of temperature on the action pattern of the amylases. In what follows, the term level of multiple attack (LMA) is used when the action pattern of an amylase is described by the alternative RV_{80}-value.

Effect of Temperature on the Action Pattern. For the different amylases, the drop in relative blue value versus the increase in reducing sugar content was determined at 35, 50, and 70 °C.

In general, the LMA of the endo-amylases increased with temperature to an extent depending on the amylase. Indeed, the LMA of TAKA and BLA increased when temperature was raised from 35 to 50 °C. In the case of BLA, hardly any further change was observed when the temperature was raised from 50 to 70 °C. The LMA of BAA and BSuA hardly changed when temperature was raised from 35 to 50 °C, while they increased more profoundly when the temperature was raised from 50 to 70 °C. For TVA, the LMA increased to a more or less similar extent when temperature was raised from 35 to 50 °C and from 50 to 70 °C. In the case of PPA, the LMA did not change significantly with temperature.

The past and the present results concerning the temperature effect on DMA seem to be inconsistent. Kramhøft and Svensson (1998)[16] found that the DMA of an α-amylase from *B. licheniformis* remained unchanged following an increase in the temperature from 37 to 80 °C. Mazur and Nakatani (1993)[14] for PPA noted a temperature dependence of the shape of the multiple attack product distribution. According to these investigators, repeated attacks are caused by movement of the enzyme along the polysaccharide chain during the lifetime of an enzyme−substrate complex, which is referred to as sliding. They suggested that the probability of sliding relative to that of dissociation increases when temperature is increased, which can be expected provided the sliding occurs in a loose complex of enzyme and substrate through hydrophobic interactions. In our work, this effect of temperature on LMA was not noted for PPA, while, for the other amylases, LMA increased with temperature. This may explain the results obtained by Marchal and coworkers (1999).[15] In their investigation, these authors found that the temperature had a distinct influence on the oligosaccharide composition after α-amylolysis of starch by α-amylases from *B. licheniformis*, *B. amyloliquefaciens*, and *B. stearothermophilus*.

The effect of temperature on the action pattern of the maltogenic amylase BStA was markedly different from that of the endo-amylases tested. When temperature was raised, LMA decreased, indicating a more pronounced endo-action of this enzyme at higher temperatures, but remained high as compared to that of the other amylases. It is of note that, both in its effect on the molecular and rheological properties of starch slurries[22,23] as well as in its action on amylose, BStA behaved in a nontypical manner. The present findings are of particular relevance for our understanding of the functionality of this enzyme in breadmaking applications. It is well known that BStA is a very potent bread antistaling enzyme. In general, its action significantly reduces the firming rate.[35−38] While it has been stated that it is mainly exo-acting,[39] our results indicate that a considerable degree of starch endo-hydrolysis may take place at temperatures exceeding those needed for wheat starch gelatinization.

From these results, it is clear that temperature influences the multiple attack action of the different amylases to a certain extent. Hence, when considering amylase functionality in food

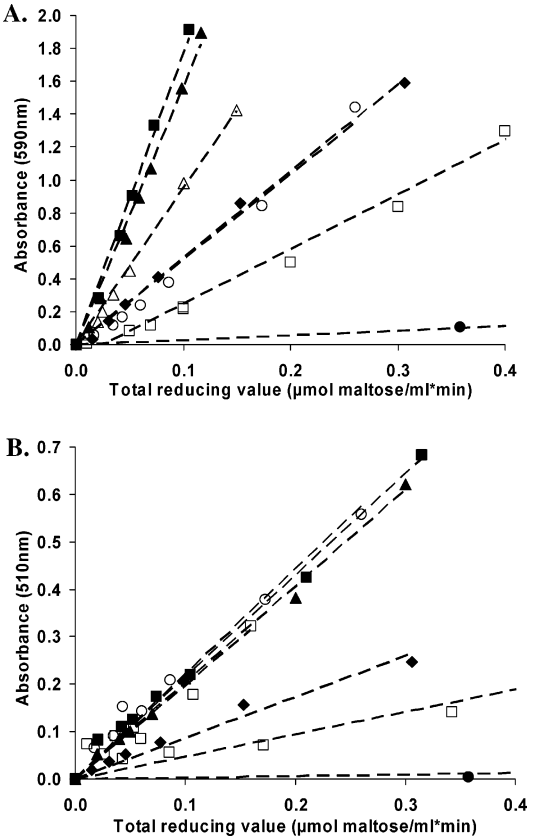

Figure 4. Action pattern of amylases at 35 °C and pH 6.0 determined using chromophoric starch substrates. (A) Azurine-cross-linked amylose (Amylazyme) as a substrate; (B) Red Starch substrate. (○) TAKA, *Aspergillus oryzae* α-amylase; (▲) BSuA, *Bacillus subtilis* α-amylase; (■) BAA, *B. amyloliquefaciens* α-amylase; (△) BLA, *B. licheniformis* α-amylase; (◆) TVA, *Thermoactinomyces vulgaris* α-amylase I; (□) PPA, porcine pancreatic α-amylase; (●) BStA, *B. stearothermophilus* maltogenic α-amylase. Experimental points are means of triplo measurements of an individual experiment out of at least three individual experiments. Standard deviations on the absorbance were less than 5%. The dashed lines shown are linear regression lines fitted to the experimental points.

systems, not only the kind of amylase with its specific DMA but also temperature will be of utmost importance.

Action Pattern with Chromophoric Substrates. Two alternative substrates were used to examine the action pattern of the amylases. The first one was azurine-cross-linked amylose (Amylazyme), which is insoluble but readily hydrates in water. The action of an endo-α-amylase on the Amylazyme substrate releases soluble colored fragments, resulting in an increase in absorbance of the supernatant. Hence, this (solubilizing) action can be considered to be a measure for the endo-action of the amylase. The second alternative substrate, Red Starch, is a soluble branched chromophoric substrate, more specifically a partially depolymerized starch, dyed with Procion Red MX-5B. When Red Starch is incubated with an α-amylase, the increase of absorbance of the supernatant following ethanol precipitation is caused by generation of low-molecular weight dyed fragments. Likewise, this action can be considered to be a measure for the endo-action of the amylase.

Figure 4 shows, for both substrates, the relation between the increase in absorbance of the supernatant versus the reducing value (expressed as the increase in reducing sugars, measured with soluble starch[20]), which is a measure for the total hydrolytic attacks, at 35 °C. For both substrates, the α-amylases could roughly be divided into three groups. BStA showed almost no solubilizing action against Amylazyme. The second group consisted of PPA, TVA, and TAKA, which showed medium solubilizing action for a certain enzyme activity. BLA, BsuA, and BAA formed the third group with a rather high solubilizing action. When Red Starch was used, similar observations were obtained, except for TAKA, which showed a rather high absorbance. From the results, it is clear that, for an equal enzyme activity on soluble starch, the various α-amylases produced different levels of soluble chromophoric fragments in the case of the insoluble Amylazyme, and different levels of low-molecular weight dyed fragments when the soluble Red Starch was used.

As indicated above, in the multiple attack action of amylases, a series of glycosidic bonds are broken during the lifetime of an enzyme−substrate complex. The first hydrolytic action of the series can be considered as a true "endo"-action. When using the chromophoric substrates, only the "endo"-action (i.e., the first bond broken) gives rise to an increase in the absorbance value by liberating a soluble, colored fragment (in the case of the Amylazyme substrate) or by generating a colored fragment of low-molecular weight (in the case of the Red Starch). In this view, the absorbance of the supernatant can be considered as a measure for these "endo"-action. Hence, for a given amylase, relating this absorbance value to the reducing value (as a measure of total hydrolytic attacks) should give an indication for its multiple attack action. This way, for a certain enzyme activity (expressed as reducing sugars generated), a low absorbance would indicate a higher apparent multiple attack, while a higher absorbance would imply a lower apparent multiple attack. In this view, BStA, PPA, TAKA, and TVA can be considered as enzymes with a high apparent multiple attack at 35 °C for Amylazyme, while for Red Starch only BStA, PPA, and TVA are detected as enzymes with a high apparent multiple attack.

For both substrates, the experimental results measured at 50 and 70 °C were also fitted with a linear regression equation and characterized by their slope. Table 2 shows that, irrespective of substrate, the slope increased for every enzyme but TAKA, when the temperature was raised.

In addition, the ratio of the slope obtained with the Red Starch substrate to that obtained with the Amylazyme substrate (further referred to as the R/X ratio) gives an indication about the relative activity of amylases toward soluble (Red Starch) and insoluble (Amylazyme) substrates, that is, their substrate selectivity (Table 2).[40] A high R/X ratio points to a preferential hydrolysis of the soluble Red Starch, while a low R/X ratio indicates that the amylase preferentially hydrolyzes the insoluble (Amylazyme) amylose. Table 2 shows that BAA (0.10) had the lowest R/X ratio, followed by BSuA, BstA, and PPA (0.12). TVA and BLA exhibited an R/X ratio of, respectively, 0.15 and 0.17, while TAKA had the highest relative activity toward the soluble Red Starch with an R/X ratio of 0.35. For all amylases but PPA, the R/X ratio decreased when temperature was raised, indicating an increased relative activity toward the insoluble substrate. At 70 °C, the R/X ratio was similar for all amylases.

Both from the hydrolysis data with potato amylose and with the chromophoric substrates, it is clear that, first, the amylases cannot be considered as hydrolyzing starch in a truly random manner and that, second, temperature has an important effect on the action pattern. However, the correlation between RV_{80}, obtained with potato amylose, and the slope obtained with the Amylazyme method at 35 °C was poor ($R^2 = 0.49$). Correlation between RV_{80}, obtained with potato amylose, and the slope obtained with the Red Starch (both at 35 °C) was better than

Table 2. Overview of Parameters Characterizing the Action Pattern of Amylases at Different Temperatures (35, 50, and 70 °C) and pH 6, Determined Using Amylazyme or Red Starch as a Substrate[a]

	slope Amylazyme			slope Red Starch			R/X		
	35 °C	50 °C	70 °C	35 °C	50 °C	70 °C	35 °C	50 °C	70 °C
TAKA	5.70	5.70	n.d.	2.00	1.75	n.d.	0.35	0.30	n.d.
BSuA	16.25	41.95	56.10	2.00	2.40	2.95	0.12	0.06	0.05
BAA	19.60	32.75	55.40	2.05	2.25	2.55	0.10	0.07	0.05
BLA	9.90	24.10	76.80	1.70	2.25	3.10	0.17	0.09	0.04
TVA	5.30	12.30	88.15	0.80	0.95	4.30	0.15	0.08	0.05
PPA	3.35	6.10	n.d.	0.40	0.85	n.d.	0.12	0.14	n.d.
BStA	0.30	0.45	5.30	0.05	0.05	0.30	0.12	0.11	0.05

[a] Slope, parameter from the linear regression estimated by the software GraphPad Prism; R/X, ratio of the slope obtained with the Red Starch substrate to that obtained with the Amylazyme substrate; TAKA, *Aspergillus oryzae* α-amylase; BSuA, *Bacillus subtilis* α-amylase; BAA, *B. amyloliquefaciens* α-amylase; BLA, *B. licheniformis* α-amylase; TVA, *Thermoactinomyces vulgaris* α-amylase I; PPA, porcine pancreatic α-amylase; BStA, *B. stearothermophilus* maltogenic α-amylase; n.d., enzyme activity could not be detected.

for the Amylazyme method, but still rather poor with $R^2 = 0.75$. Moreover, for both chromophoric substrates, the apparent multiple attack decreased when temperature was raised, while with the potato amylose substrate it increased (except for BStA, as discussed before). The correlation between RV_{80} and the slope obtained with the Amylazyme ($R^2 = 0.58$ at 50 °C and 0.81 at 70 °C) and Red Starch substrate ($R^2 = 0.77$ at 50 °C and 0.83 at 70 °C) was better at higher temperatures. The presence of dyed substituents in cross-linked amylose (Amylazyme) and Red Starch can be responsible for the poor correlation with the results obtained for potato amylose. Differences in the R/X ratio of the amylases are probably also one of the reasons for the poor correlation, especially at 35 °C. Indeed, when the temperature was raised, the differences in R/X ratio became less pronounced with a better correlation as a result. Also, the fact that both Red Starch and amylose were soluble under the experimental conditions used can explain the better correlation between the results obtained with these two substrates. Hence, the high relative activity toward soluble Red Starch of TAKA probably explains why a rather high multiple attack action was obtained for TAKA using the Amylazyme assay, while TAKA had the lowest DMA when using potato amylose. This clearly demonstrates that the action pattern of α-amylases is influenced by the nature of the substrate (solubility, molecular weight, presence of substituents and side chains). Consequently, it can be expected that DMA on amylopectin will differ from DMA on amylose. Further experimental work with amylopectin will be necessary. Furthermore, this also implies that the cross-linked amylose (Amylazyme) and Red Starch are ill suited as alternative substrates to estimate the DMA of an amylase.

Conclusion

Different amylases had a different DMA on amylose. The maltogenic BStA had the highest DMA, followed by PPA. BLA, BAA, TVA, and BSuA had intermediate and TAKA had the lowest DMA. An alternative procedure for the laborious DMA assay was proposed. In the alternative experimental method, the level of multiple attack (LMA) was determined from the relation between the drop in relative blue value versus the increase in reducing sugar content as the RV_{80}-value (i.e., the total reducing value corresponding to a relative blue value of 80%).

The RV_{80}-values at 35, 50, and 70 °C demonstrated that temperature influences amylase action pattern. For the endo-amylases tested, LMA in general increased when temperature was raised, with the extent of the LMA increase depending on the endo-amylase. Remarkably, the maltogenic BStA showed an enhanced endo-action (i.e., a decreased LMA) with increasing temperature. Because amylases are used to alter starch structure and, consequently, the physicochemical behavior of starch, these results imply that the application of amylases with various DMA and the influence of temperature on DMA might be useful tools for the development of tailor-made starch.

Not only the temperature but also the structural features of the substrate impacted the action pattern of the different amylases. Hence, in understanding amylase functionality in food systems, it is of utmost importance to consider that their DMA and, hence, their functionality vary not only with temperature but are also influenced by structural parameters of the starch substrate.

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