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Antifirming Effects of Starch Degrading Enzymes in Bread Crumb

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Antifirming properties of amylases in bread crumb were evaluated in straight dough breadmaking and related to the amylolytically modified starch structure. Amylase properties and action mechanisms determine starch structure in the breads and, hence, how amylopectin recrystallization, starch network formation, water redistribution, and water mobility occur during breadmaking and storage. A bacterial endo-α-amylase mainly hydrolyzed the longer starch polymer chains internally. It thus reduced the number of connections between the crystallites in the starch networks, resulting in a softer bread crumb. However, because the enzyme had only little impact on the outer amylopectin chains, amylopectin recrystallization and the concomitant water immobilization presumably were not hindered. The loss of plasticizing water as a result of recrystallization presumably reduces the flexibility of the gluten network and results in poor crumb resilience. In contrast, in breadmaking, the *Bacillus stearothermophilus* maltogenic α-amylase acted as an exoacting amylase with more pronounced endoaction at higher temperatures. This enzyme caused extensive degradation of the crystallizable amylopectin side chains and thus limited amylopectin recrystallization and network formation during storage. As a result, it prevented the incorporation of water in the amylopectin crystallites. In this way, the different starch and gluten networks kept their flexibility, resulting in a softer crumb with good resilience.

KEYWORDS: Amylase; starch structure; bread staling; amylopectin recrystallization

INTRODUCTION

In the Western hemisphere, bread has a place in most people's daily diet. Despite its importance, the qualitative understanding of physical processes occurring during breadmaking and storage is still limited (*1*). In particular, bread staling, of which crumb firming and loss of crumb resilience are an integral part, is an important problem. Hence, considerable (research) efforts have been and are devoted to understanding the firming of bread crumb during storage as well as to increasing bread shelf life. A thorough overview of the role of the different players involved in crumb firming during storage and means to prevent it can be found elsewhere (see, e.g., refs *2* and *3*).

It is widely accepted that rearrangements in the starch fraction, usually referred to as starch retrogradation, play an important role in the crumb firming process. Starch retrogradation is defined as the reassociation of the starch polymers during and after cooling of a gelatinized starch paste to a more ordered or crystalline state (*4*). However, the amylose and amylopectin populations behave differently. Therefore, we will refer to the terms amylose gelation and crystallization and amylopectin recrystallization to describe the starch reassociations occurring during cooling and storage of bread. We do this because, from an etymological as well as a historical perspective, the term retrogradation (which means going back to an original state) was first introduced to describe the fact that native crystalline starch following gelatinization, cooling, and storage regains crystallinity. As in native starch only amylopectin structural elements are crystalline, the term retrogradation is best reserved for amylopectin crystallization (*5*).

It is also well-known that some (bacterial) α-amylases decrease the rate of bread firming. During baking, amylases partially hydrolyze starch molecules, generating dextrins in situ. Their impact on the starch molecules inter alia depends on their temperature window of activity in the baking phase, the accessibility of the starch polymers in the granules, their mode of action, and the enzyme dosage. Several authors have put forward mechanisms by which amylases decrease bread firmness. In one view, their antistaling properties are attributed to the changed (re)crystallization behavior of the residual amylose and amylopectin populations (*2*, *6*, *7*). Alternatively, other studies reported that the formed dextrins may interfere with gluten−starch interactions (*8*) or with the reassociation of the residual starch fraction (*9*, *10*), thus reducing the rate of bread firming. However, most of the latter studies were based on addition of dextrins to bread dough and often failed to take the consequences of this setup into account. Indeed, dextrin addition affects dough water absorption (*11*), whereas high levels of

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dextrins delay starch gelatinization (*12*, *13*). Finally, added dextrins can be hydrolyzed by endogenously present β-amylases and added α-amylases (*6*) and can then largely be consumed by the yeast.

The different views on the antifirming mechanism of amylases reflect the still incomplete understanding of the firming mechanism itself. In the present study, the impacts of different amylases on crumb texture, and on amylopectin recrystallization, were evaluated and correlated with the structural properties of the residual amylopectin molecules. The latter were analyzed using size exclusion chromatography (SEC) and, following debranching, by high-performance anion-exchange chromatography (HPAEC). The amylases used in the present study represent enzymes with different thermal stabilities and modes of action. To the best of our knowledge, such an integrated approach and, especially, the determination of the structure of starch in breads made with the addition of different amylases, have not been used elsewhere in investigations of the antifirming functionality of amylases. The results are discussed within the framework of a bread staling model.

MATERIALS AND METHODS

Materials. Flour samples were milled from the wheat variety Legat on a Bühler MLU-202 laboratory mill (Uzwil, Switzerland) according to AACC Method 26-31 (*14*). Moisture content was determined on the basis of weight loss at 130 °C for 120 min of ca. 1.0 g of accurately weighed samples (AACC Method 44-15A) (*12*) and was 13.8%. Starch content of the flour was determined using a gas chromatographic procedure following hydrolysis and conversion to alditol acetates (*15*) and was 76.8%. The level of damaged starch [Starch Damage Assay Kit (Megazyme, Bray, Ireland); AACC Method 76-31] (*14*) was 4.5%. Protein content [11.3% on dry matter (dm)] was determined by the Dumas method (N × 5.70), an adaptation of the AOAC Official Method (*16*), using an automated Dumas protein analysis system (EAS VarioMax N/CN, Elt, Gouda, The Netherlands). All chemicals and reagents were from Sigma Aldrich Chemie (Bornem, Belgium), unless stated otherwise.

Enzymes. *Bacillus subtilis* endo-α-amylase (BSuA) and protease-free porcine pancreatic endo-α-amylase (PPA) were from Sigma Aldrich Chemie. *Bacillus stearothermophilus* maltogenic amylase (BStA, trade name Novamyl) was from Novozymes (Bagsvaerd, Denmark). It is an effective antifirming enzyme widely used in breadmaking. The enzymes used were free of interfering starch-degrading activity, as checked by size exclusion chromatography [using a Hiprep Sephacryl S100 (26 × 670 mm) (GE Healthcare, Uppsala, Sweden)] and activity measurements (*17*). Amylase activities were assayed by quantifying the reducing sugars released from soluble starch [1.0% (w/v) solution] (Merck, Darmstadt, Germany) according to the Somogyi−Nelson method (*18*), using a maltose standard curve. One enzyme unit (1 EU) is the amount of enzyme that releases 1 μmol of maltose/min at 40 °C and pH 6.0 (100 mM sodium maleate buffer containing 5.0 mM $CaCl_2$). BStA dosages of 5.05 and 10.1 EU/g of flour correspond to earlier recommended dosages for breadmaking (*19*). BSuA dosages of 0.10 and 0.12 EU/g of flour were used. In a rapid visco analysis (RVA) study on starch slurries, these BSuA dosages induced a similar peak viscosity as did the recommended dosages of BStA (*20*). Higher enzyme dosages (BStA, 20.2 EU/g of flour; BSuA, 0.17 EU/g of flour) were also applied to increase the impact on the starch population. The ca. 50−100 times more enzyme units used for the maltogenic BStA than for the endoacting BSuA to be suitable for the purpose of this study reflect the difference in mode of action of these enzymes, as was also the case in the study on amylase-supplemented starch slurries (*17*, *20*). In the case of PPA, it was essential to use a high dosage (68.4 EU/g of flour) in the breadmaking experiments, because of strong inhibition of this enzyme by endogenous wheat proteins belonging to the cereal trypsin/α-amylase inhibitor families (*21*) and its low thermal stability.

Other enzymes needed for the preparation and analysis of the starch polymer fractions included trypsin and papain, both devoid of amylases (Sigma-Aldrich Chemie), a *Pseudomonas* isoamylase (Megazyme), and barley β-amylase (Megazyme).

Breadmaking Procedure. Dough (4.0 kg) was prepared from flour (1000 parts), compressed yeast (Bruggeman, Ghent, Belgium; 53 parts), 5.0 mM $CaCl_2$ solution (590 parts), sugar (60 parts), and salt (15 parts). The ingredients were mixed for 5.5 min at 20 °C in a spiral mixer (De Danieli, Legnaro, Italy). The dough was then divided into pieces of 450 g and fermented for 90 min (at 35 °C and relative humidity of 95%) with intermediate and final punching at 52, 77, and 90 min, respectively. Following molding, dough was proofed (36 min) and baked at 210 °C for 40 min in a rotary oven (National Mfg., Lincoln, NE) (*22*). Bread was cooled for 240 min before loaf volume measurement by rapeseed displacement. Next, the breads were weighed and packed in hermetically sealed plastic bags to prevent moisture loss. The coefficient of variation for the specific loaf volumes was <2.5%. Breadmaking experiments performed on different days included a control (amylase-free) dough.

Crumb Firmness and Resilience. Bread firmness was quantified as described in AACC Method 74-09 (*12*), using a TA.XT2 instrument (Stable Microsystems, Surrey, U.K.) with a 40 mm diameter cylindrical probe at a test speed of 1.0 mm/min (*23*). The sample was compressed by 25%. The average values based on three different loaves are reported. For each loaf, at least four slices of bread (25 mm thickness each), cut from the loaf center, were analyzed. The resilience (elastic recovery) of the bread samples was evaluated manually by an experienced baker, relative to that of a control bread sample.

Digital Image Analysis. To study the crumb structure, a single 40 × 40 mm field of view capturing the crumb area of the center of each scanned bread slice (full scale in 256 gray levels at 300 dots per inch) was processed using the image processing toolbox of Matlab 6.1 (The Mathworks, Natick, MA) as described by Lagrain and co-workers (*22*).

Differential Scanning Calorimetry (DSC). DSC measurements were performed with a DSC Q1000 (TA Instruments, Newcastle, U.K.). Recrystallized amylopectin levels in the bread samples were analyzed after 0 and 6 days of storage, using 30−40 mg of crumb (accurately weighed) without water addition, in DSC high-pressure pans (Mettler Toledo, Norwalk, CT). The pans were sealed, equilibrated at 0 °C in the DSC, and heated from 0 to 150 °C at a heating rate of 4 °C/min. Before analysis, the system was calibrated with indium, and an empty pan was used as reference. The melting temperatures and enthalpies corresponding to the melting of recrystallized amylopectin and the dissociation of amylose−lipid complexes were evaluated from the thermograms using Universal Analysis (TA Instruments). The enthalpy was expressed in joules per gram of crumb (on dry basis) (*23*).

Hot-Water Extractable Dextrin Content of Bread Crumb. Dextrins (soluble starch) were extracted from control and amylase-treated loaves at 95 °C (*6*). In this procedure, 1.5 g of bread crumb was suspended in 25.0 mL of hot water (95 °C, 20 min) under mild shaking. The supernatant after centrifugation (10 min, 3000*g*) was analyzed for total glucose (and referred to as hot-water extractable dextrin content) and average degree of polymerization (DP) of the obtained dextrin fraction. The latter was calculated from the molar ratio of total carbohydrate to reducing end contents, such as measured with the phenol−sulfuric acid (*24*) and Somogyi (*18*) methods, respectively.

Size Exclusion Chromatography (SEC). Freeze-dried bread crumb samples were milled in a coffee grinder and sieved (400 μm sieve). A flour sample and the bread crumb samples (10 mg) were dissolved in 1.0 mL of 1.0 M KOH for 5 h under mild magnetic stirring and then diluted to 10.0 mL with demineralized water. After filtration (0.45 μm; regenerated cellulose syringe filter), 5.0 mL of the filtrates was fractionated using a Sepharose CL-2B column (74 cm × 1.6 cm, GE Healthcare) and 0.1 M KOH as eluent (*20*, *25*). The amylose molecular weight (MW) distribution was visualized using postfractionation complexation with KI/I_2 solution (0.38 mg of I_2/mL and 0.90 mg of KI/mL) and absorbance measurement at 620 nm as described earlier (*20*). The residual amylose fraction in the amylase-supplemented bread

samples was quantified by comparing the integrated KI/I_2 absorbance values of the eluates with those of the control sample. The polydispersity (P) is the ratio of the weight-average DP to the number-average DP (*26*).

Isolation of Starch from Bread Crumb. Starch was isolated from the freeze-dried and subsequently ground bread crumb samples, taken 4 h after baking, following enzymic degradation of the protein fraction. In this procedure, 25.0 mL of water was added to 10.0 g of bread crumb, and the mixture was incubated for 30 min at 95 °C to inactivate the residual amylases. Next, 100 mL of a protease solution, containing 0.50 g of trypsin and 0.25 g of papain, was added to degrade the gluten network. After incubation (4 h, magnetic stirring) at ambient temperature, the proteases were inactivated (15 min, 95 °C) and the suspension was centrifuged (10 min, 3000*g*). The residues were washed twice at ambient temperature with 10.0 mL of ethanol (95%). Protein contents of the isolated starch fractions were determined according to the above Dumas method. None of the samples had a protein content exceeding 2%. The isolated starch was analyzed by SEC as above.

Starch Fractionation: Isolation of Amylopectin. The isolated starch was fractionated into amylose and amylopectin as described by Leman and co-workers (*17*), using preferential precipitation of amylose with 1-butanol and isoamyl alcohol. The purity of the isolated amylopectin fractions was analyzed using SEC (on a Sepharose CL-2B column, see above) with subsequent iodine binding analysis of the eluates (*17*). The amylose fractions were not further analyzed.

Debranching of the Isolated Residual Amylopectin. Enzymically debranched amylopectins were prepared following incubation with a *Pseudomonas* isoamylase based on the procedure of Klucinec and Thompson (*25*) as described earlier (*17*). Debranching was considered to be complete when longer incubation times with the isoamylase did not further increase the reducing capacity of the digest determined according to Somogyi (*18*). The preparation of the debranched amylopectin samples was performed in triplicate. Following enzymic debranching, the average chain length (CL) of the residual amylopectin fractions was calculated as the molar ratio of total carbohydrate to reducing end contents, measured with phenol−sulfuric acid (*24*) and Somogyi (*18*) methods, respectively. For each debranched amylopectin sample, these analyses were performed in triplicate.

Analysis of Amylopectin Branch Chain Length Distributions. The enzymically debranched amylopectins were analyzed by anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) on a Spectra system P4000 (Thermo Electron Corp., Waltham, MA) system, using Carbopac PA Guard and Carbopac PA-100 (250 mm × 4 mm) columns. The pulse potentials and durations, the eluents, and the eluent gradient program were as described by Jacobs and co-workers (*27*). Individual peaks in the chromatograms were corrected for molar PAD detector responses (*28*). Analyses were performed at least in duplicate. The relative levels of amylopectin chains were normalized and divided into six fractions, corresponding to degrees of polymerization (DP) ≤ 9, DP 10−11, DP 12−16, DP 17−24, and DP ≥ 25, based on an earlier study on rice starch (*29*).

Preparation of β-Limit Dextrins of the Isolated Amylopectin. The β-limit dextrins of the purified amylopectins were prepared according to the method of Klucinec and Thompson (*30*), with some modifications described by Leman and co-workers (*17*). In short, amylopectin (12.0 mg) was first dispersed in 120 μL of 90% DMSO by heating (10 min, 100 °C), and sodium acetate buffer (0.02 N; pH 6.0; 880 μL; 50 °C) was then added. Barley β-amylase solution [0.02 N sodium acetate, pH 6.0; 50 μL; 250 U/mL (units as defined by the supplier)] was added to hydrolyze the external chains of the residual amylopectin. The reaction proceeded at 50 °C during a first incubation for 48 h, and, following enzyme inactivation and addition of fresh barley β-amylase solution as above, during a second incubation for 22 h. Under such conditions, the β-amylolysis was considered to be complete because longer incubation times with β-amylase did not further increase the reducing capacity of the digest when determined according to Somogyi (*18*). The β-limit dextrins were precipitated by mixing a 0.5 m/L aliquot of each digest with 1.5 mL of 95% ethanol, recovered by centrifugation (10 min, 250*g*) and, following two washing steps with ethanol (95%) and one with acetone, dried in a forced-air oven (50 °C, 30 min).

Table 1. Loaf Volume and Crumb Firmness of Breads with or without Addition of Amylases at Various Concentrations in the Breadmaking Recipe[a]

amylase (concn, EU/g of flour)	loaf vol (cm^3)	crumb firmness (N)	
		day 0	day 6
control	1053 ± 18 a	3.15 ± 0.25 a	16.85 ± 0.25 a
BSuA (0.10 EU)	1070 ± 14 a	3.40 ± 0.20 a	10.55 ± 0.05 b
BSuA (0.12 EU)	1107 ± 22 a	3.15 ± 0.05 a	8.95 ± 0.10 bc
BSuA (0.17 EU)	1124 ± 9 b	3.25 ± 0.25 a	7.35 ± 0.20 cd
PPA (68.4 EU)	1090 ± 26 a	2.95 ± 0.10 a	15.90 ± 0.35 a
BStA (5.05 EU)	1023 ± 19 a	5.55 ± 0.25 c	9.00 ± 0.20 bc
BStA (10.1 EU)	1063 ± 34 a	4.95 ± 0.35 b	7.15 ± 0.25 d
BStA (20.2 EU)	1102 ± 20 a	3.10 ± 0.10 a	4.40 ± 0.10 e

[a] Values followed by different letters in the same column indicate significantly different means at $P < 0.05$. BSuA, *Bacillus subtilis* α-amylase; PPA, porcine pancreatic α-amylase; BStA, *Bacillus stearothermophilus* maltogenic α-amylase.

The β-amylolysis limit (β-limit) was calculated as the molar ratio of reducing ends after incubation with β-amylase [determined according to Somogyi (*18*) using a maltose reference curve] to total carbohydrate contents [quantified according to the phenol−sulfuric acid method (*24*)]. Assuming that every constituent amylopectin chain is accessible and that every chain is (partially) hydrolyzed by β-amylase, one can calculate the average length of the portion of the chains that is external to the branch points, referred to as the average outer chain length (OCL) (*31, 32*), as

$$\mathrm{OCL} = \mathrm{CL} \times (\beta\text{-limit}) + 2$$

Statistical Analyses. For statistical analyses, the t test (PROC ANOVA) was used (significance level $P < 0.05$). Statistical analyses were conducted using Statistical Analysis System software 8.1 (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Bread Properties. The firming of bread crumb and its lower resilience are essential factors in bread quality losses upon storage. **Table 1** lists the loaf volume of the different bread samples. The addition of the amylases to the recipe overall had little if any influence on the bread volumes, which mainly ranged from 1050 to 1100 cm^3, except in the case of bread to which BSuA (0.17 EU/g of flour) was added, which had a slightly higher volume. Loaf volume will hence not be a major factor contributing to differences in crumb hardness of the different samples. The crumb structures of the BStA- and PPA-supplemented breads were similar to that of the control bread, whereas the BSuA-supplemented breads had a coarser crumb structure, in agreement with Lagrain and co-workers (*22*). Bread weight did not change during storage, demonstrating that the use of sealed plastic bags prevented water loss (results not shown). Furthermore, during storage, moisture content in the crumb decreased from 44−45 to 40−42%, indicating that water had migrated from the bread crumb to the crust. This occurred to similar extents for the breads with and without amylase addition (results not shown). The addition of the different amylases led to acceptable bread qualities, except in the case of high dosages of BSuA (0.17 EU/g of flour) and BStA (20.2 EU/g of flour), which led to sticky doughs and a low resilience (as evaluated manually). Therefore, higher levels of the bacterial enzymes, particularly of BSuA, were not suitable for breadmaking purposes.

Crumb Firmness and Resilience and Recrystallized Amylopectin Levels. Table 1 lists crumb firmness data obtained 4 h after baking (day 0, d0) and after 6 days (d6) of storage at ambient temperature. Under the experimental conditions, the initial firmness of the control breads at d0 was 3.15 N. Neither

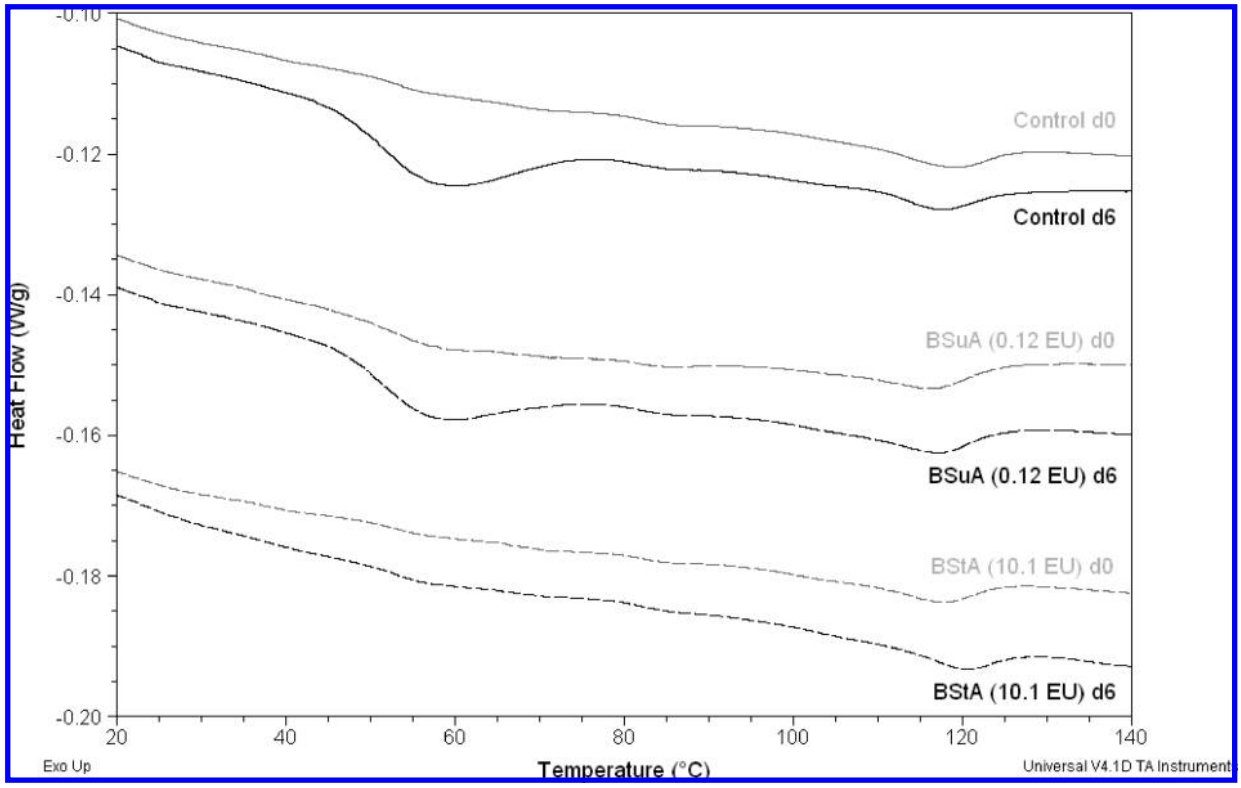

Figure 1. Typical DSC thermograms of crumb samples from control and *B. subtilis* (BSuA) and *B. stearothermophilus* (BStA) amylase-supplemented breads after cooling (d0) and after 6 days of storage (d6).

BSuA nor PPA had any significant influence on the initial crumb firmness. BStA addition (5.05 and 10.1 EU/g of flour) increased the initial firmness readings (5.55 and 4.95 N, respectively), except at the highest (over)dosage (20.2 EU/g of flour) (3.10 N). The higher initial firmness of the BStA samples is in line with literature data (*7*) and can be related to the higher end viscosity observed in RVA analysis of starch slurries supplemented with BStA (*17*). During storage for 6 days, the firmness of the control bread increased to 16.85 N, whereas crumb resilience decreased. The enzymes affected firming differently. BSuA addition reduced the d6 firmness to 10.55, 8.95, and 7.35 N for dosages of 0.10, 0.12, and 0.17 EU/g of flour, respectively. However, the crumb resiliences were inferior to that of the control and further decreased during storage. Despite the high enzyme activity level added, PPA addition (68.4 EU/g of flour) resulted in only a slight (5%) decrease in crumb firmness at d6. BStA addition reduced the firming rate drastically. Firmness at d6 was 9.00, 7.15, and 4.40 N for BStA dosages of 5.05, 10.1, and 20.2 EU/g of flour, respectively; these values are considerably lower than the control values. In contrast to BSuA, BStA-supplemented breads had a good crumb resilience. This remained so during storage: the d6 resilience of BStA breads was even superior to that of the control. Our crumb firmness data and resilience evaluation are generally in good agreement with literature data on these parameters in maltogenic α-amylase and conventional bacterial endo-α-amylase-supplemented breads (*7*).

Recrystallized amylopectin levels were estimated by DSC analysis of bread crumb. **Figure 1** shows typical DSC thermograms of control and amylase-supplemented crumb samples at d0 and d6. **Table 2** lists the melting temperature and enthalpy values of the recrystallized amylopectin in the d6 crumb samples. As can be seen in **Figure 1**, for all d0 samples, very low endothermic signals were observed between 50 and 80 °C, which can presumably be attributed to the melting of the very low levels of recrystallized amylopectin in these samples. The main peak in the d0 samples corresponds to the dissociation of amylose lipid complexes ($T_p \sim 120-130$ °C). Amylase supplementation had little if any impact on the levels of amylose−lipid complexes in the breads.

Table 2. Onset (T_o), Peak (T_p), and Conclusion (T_c) Temperature and Enthalpy (ΔH_{AP}) Values of Melting of Recrystallized Amylopectin in Bread Samples with or without Addition of Amylases at Various Concentrations in the Breadmaking Recipe, Analyzed by DSC after 6 Days of Storage[a]

amylase (concn, EU/g of flour)	T_o (°C)	T_p (°C)	T_c (°C)	ΔH_{AP} [J/g of crumb (dm)]
control	47.5 ± 0.9	59.5 ± 1.1	76.4 ± 1.1	2.46 ± 0.18 a
BSuA (0.10 EU)	48.2 ± 0.7	59.0 ± 0.5	76.3 ± 1.0	2.02 ± 0.10 b
BSuA (0.12 EU)	48.2 ± 0.9	58.4 ± 1.1	76.6 ± 0.9	1.97 ± 0.07 b
BSuA (0.17 EU)	48.1 ± 0.9	59.2 ± 0.9	76.8 ± 1.2	1.45 ± 0.15 c
PPA (68.4 EU)	47.3 ± 0.5	58.5 ± 0.8	75.1 ± 0.5	2.16 ± 0.13 b
BStA (5.05 EU)	49.7 ± 1.0	59.0 ± 0.9	77.9 ± 0.9	0.63 ± 0.10 d
BStA (10.1 EU)	49.5 ± 0.4	58.7 ± 1.0	77.6 ± 0.8	0.47 ± 0.09 d
BStA (20.2 EU)	49.9 ± 0.6	58.6 ± 1.30	78.4 ± 1.0	0.31 ± 0.04 e

[a] Values followed by different letters in the same column indicate significantly different means at $P < 0.05$. BSuA, *Bacillus subtilis* α-amylase; PPA, porcine pancreatic α-amylase; BStA, *Bacillus stearothermophilus* maltogenic α-amylase.

After 6 days of storage, in addition to the peak corresponding to amylose−lipid complex dissociation, a major peak ($T_p \sim 58-60$ °C), attributed to recrystallized amylopectin melting, was observed for the control and the endo-α-amylase-containing crumb samples (**Figure 1**). Amylase supplementation had in general little if any effect on the melting temperatures of the recrystallized amylopectin (**Table 2**). For the control bread, this resulted in a melting enthalpy of 2.46 J/g at d6. BSuA addition led to lower melting enthalpy values of recrystallized amylopectin (ca. 1.5−2.0 J/g), whereas PPA addition reduced the level of recrystallized amylopectin to a limited extent. In contrast, BStA almost completely suppressed the amylopectin recrystallization even after a storage time of 6 days. Indeed,

Table 3. Hot-Water Extractable Dextrin Content and Average DP from Crumb Samples with or without Addition of Amylases at Various Concentrations in the Breadmaking Recipe[a]

	day 0		day 6	
sample/amylase (concn, EU/g of flour)	recovery [%/g of bread (dm)]	av DP	recovery [%/g of bread (dm)]	av DP
control	9.7	2.1	10.0	2.0
BSuA(0.10 EU)	17.9	5.1	22.9	5.4
BSuA(0.12 EU)	21.2	6.2	27.1	nd
PPA(68.4 EU)	14.2	5.5	14.3	5.3
BStA(5.05 EU)	21.6	2.8	21.9	2.8
BStA(10.1 EU)	22.3	2.6	22.2	2.7

[a] BSuA, *Bacillus subtilis* α-amylase; PPA, porcine pancreatic α-amylase; BStA, *Bacillus stearothermophilus* maltogenic α-amylase.

the DSC thermograms at d6 were very similar to those at d0 (**Figure 1**), resulting in 4 (BStA 5.05 EU/g of flour) to 8 (BStA 20.2 EU/g of flour) times lower melting enthalpies of recrystallized amylopectin than of the control bread. These results are in agreement with literature data (*7*).

Starch Properties and Amylase Action. *Analysis of the Hot-Water Extractable Dextrins.* The hot-water extractable dextrins made up ca. 10% [dry matter (dm)] of the fresh and aged control breads. They had an average DP of about 2 (**Table 3**). Upon addition of BSuA (0.10 and 0.12 EU/g of flour), about 18 and 21% (dm) of hot water-extractable starch fragments were detected, with average DP values of 5.1 and 6.2, respectively. After 6 days of storage, these levels had increased up to ca. 23 and ca. 27% (dm), respectively. This increase shows that BSuA remains active after breadmaking, in line with earlier findings (*22*). The PPA-supplemented bread had a slightly increased dextrin content [14% (dm)], which was not altered during storage. The addition of BStA (5.05 and 10.1 EU/g of flour) increased the total level of hot-water extractable dextrins to ca. 22% (dm), with an average DP of 2.6−2.8. This did not change during storage.

Molecular Size Distribution of the Starch (Amylose) Polymers. SEC analysis of the freeze-dried crumb samples at d0 and d6 was performed to better understand the impact of the (enzymically modified) starch polymers on bread characteristics. Under the experimental conditions, amylopectin eluted in the void volume. The starch in the Legat flour had a very broad, polydisperse amylose fraction ($P = 2.2$), with peak DP of ca. 2200 (results not shown). After control breadmaking, the SEC profile was little affected, although a reduced MW of the amylose fraction was observed, with a peak DP of ca. 1100 ($P = 2.2$), which is probably mainly due to endogenous enzyme action. During storage, the amylose population showed little if any further changes (results not shown).

BSuA supplementation resulted in partial hydrolysis of the starch fraction in the breads (**Figure 2**). The area under the curve corresponding to the amylopectin peak was markedly reduced by this enzyme. BSuA (0.10 and 0.12 EU/g of flour) affected the amylose fraction to a large extent as well, resulting in a population of increased monodispersity ($P = 1.8$ and $P = 1.6$) and a peak DP of ca. 800. In addition, at these BSuA dosages, only 65 and 58%, respectively, of the amylose in the control sample could be detected using postfractionation KI/I_2 complexation. As already outlined above, we again found that BSuA clearly remained active after breadmaking, resulting in a continued degradation of the starch polymers during storage, as evidenced by a decreased KI/I_2 complexation recovery and slightly lower peak MW at d6. PPA addition reduced the amylopectin peak to a lesser extent than BSuA addition and had only little effect on the amylose population compared to that of the control bread (results not shown). The starch fraction was not further degraded during storage. The addition of the maltogenic amylase BStA degraded the starch molecules significantly (**Figure 3**). The reduced area under the curve corresponding to amylopectin demonstrated the amylopectin degradation. However, this was less pronounced than was the case with BSuA. At all concentrations studied, the amylose fraction was degraded to a large extent as well, resulting in rather monodisperse populations (*P* ranging from 1.3 to 1.8) with peak DPs ranging from ca. 500 to ca. 800 and amylose recoveries (estimated by the integrated KI/I_2 complexation values) of 52−70%. Although the enzyme is not inactivated during baking (*22*), the molecular size of the starch molecules was only slightly altered during storage, as evidenced by the SEC chromatograms after 6 days of storage (**Figure 3**). Thus, although residual BStA activities can be measured in bread crumb extracts (*22*), the enzyme, which is highly active at high temperatures and which is inhibited by maltose (*33*), does not further hydrolyze starch at bread storage temperatures.

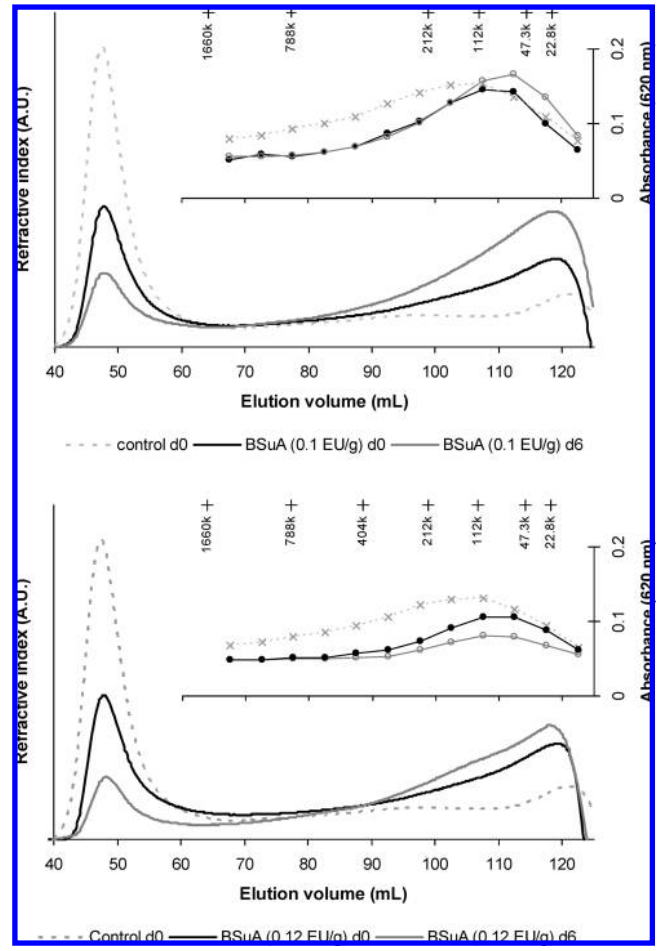

Figure 2. Size exclusion chromatography elution profiles of crumb samples from control and *B. subtilis* α-amylase supplemented breads after cooling and after 6 days of storage. Enzyme concentrations: 0.1 EU/g of flour (top) and 0.12 EU/g of flour (bottom). (Inset) Amylose molecular weight distribution as analyzed by KI/I_2 absorbance measurements (620 nm) of the eluted fractions.

Amylopectin Fine Structure and Unit Chain Length Distribution. Starch was isolated from the crumb of the different breads following proteolytic degradation of the gluten network. Crumb samples were taken only at day 0. Starch structure will presumably little change during storage in the case of PPA, which is inactivated during baking, and BStA, which has

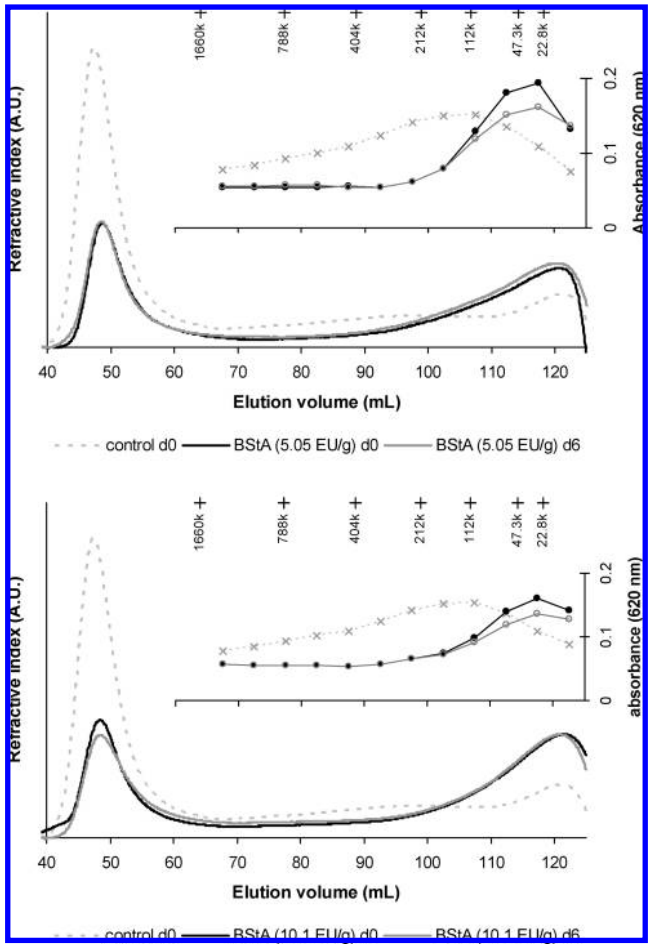

Figure 3. Size exclusion chromatography elution profiles of crumb samples from control and *B. stearothermophilus* maltogenic α-amylase supplemented breads after cooling and after 6 days of storage. Enzyme concentrations: 5.05 EU/g of flour (top) and 10.1 EU/g of flour (bottom). (Inset) Amylose molecular weight distribution as analyzed by KI/I_2 absorbance measurements (620 nm) of the eluted fractions.

neglectable levels of activity at room temperature and is inhibited by maltose (*22*, *33*). In the case of BSuA, further degradation during storage can presumably be extrapolated from the changes occurring during baking (*22*). Almost all glucose was recovered in either the residual starch or the soluble dextrin fractions. Protein content of the isolated starch fractions was <2% (dm). Following fractionation of starch in amylose and amylopectin, the purity of the isolated amylopectin fractions was evaluated using SEC and postfractionation KI/I_2 complexation.

At least 95% of the glucose present in the residual starch fraction was recovered in the respective amylose and amylopectin fractions. **Figure 4** shows typical SEC chromatograms of the control and amylase-supplemented isolated starch and amylopectin samples compared to those of the respective crumb sample. The starch and crumb profiles are generally highly similar, indicating that no further degradation took place during isolation. Likewise, the amylopectin profile does not indicate further starch degradation, but clearly shows lower levels of low MW material. In addition, we found low iodine complexation values for the eluted fractions after SEC analysis of the amylopectin samples, which indicated the absence of amylose. These data demonstrated that highly "pure" amylopectin fractions were obtained. The high recovery, the high purity, and the similar amylopectin SEC elution profiles of the starch and the corresponding isolated amylopectin fractions are good indications that the isolated amylopectin fractions represent the amylopectin population as it occurs in the bread crumb.

The isolated amylopectin fractions were debranched using isoamylase, and their chain length distributions were visualized by HPAEC-PAD (**Figure 5**). The different side chains were grouped. **Table 4** summarizes the respective area percentages for the different samples. **Table 5** lists CL, β-limit, and OCL values describing the (average) amylopectin fine structure.

A large level of the amylopectin of the control bread sample consisted of chains with a DP of 9−12. Indeed, DP ≤ 9, DP 10−11, and DP 12−16 accounted for ca. 36, ca. 19, and ca. 27%, respectively, of the total molecule. Compared to the side-chain distribution of wheat starch that was not subjected to a breadmaking procedure (*17*), there was a clear shift toward shorter side chains for the amylopectin of the control bread sample. As for amylose, this is presumably due to the action of flour enzymes. After breadmaking, the CL of the control sample amylopectin corresponded to a DP of 22.5, whereas its β-amylolysis limit was 48.6%.

In general, the amylase action on amylopectin during breadmaking was most notable by changes in the region with DP < 12 (**Figure 5**; **Table 4**). Irrespective of the dosages used, the side-chain distributions of amylopectin from the BSuA-treated samples were similar to those of the control bread amylopectin sample. Presumably, this can be related to both the rather low enzyme activity levels added and the action pattern of the enzyme (see below). Likewise, CL and β-limit (and hence also OCL) values of the residual amylopectin in the BSuA-supplemented breads (0.10 and 0.12 EU/g of flour) were similar to those of the control bread amylopectin sample. Only overdosing BSuA (0.17 EU/g of flour) significantly decreased CL (DP 11.1) and β-limit (38.7%) values of the recovered amylopectin fraction (**Table 5**). The very limited changes in the side-chain profile are somewhat in contrast to the large decrease in the area under the curve representing the residual amylopectin fraction, as seen in the Sepharose Cl-2B chromatograms (**Figure 1**). In general, the impacts noted correspond well with those of BSuA addition on amylopectin structure during RVA analysis (*17*).

PPA supplementation slightly altered the chain length distribution of the residual amylopectin fraction (**Figure 5**). It seems that PPA mainly acted on the shortest amylopectin chains, because it slightly reduced the relative level of the chains with DP ≤ 9, whereas it slightly increased the relative level of longer chains (**Table 4**). The impact of PPA on the amylopectin CL was also limited, whereas the β-limit (44.8%) and OCL (DP 11.9) values were slightly lower than those of the control bread amylopectin sample. Somewhat similar trends were seen upon PPA addition in RVA analysis (*17*).

BStA had the most drastic impact on the side-chain distribution of the residual amylopectin fraction and clearly shifted the curves to lower MW (**Figure 5**; **Table 4**). The relative level of amylopectin chains with DP ≤ 9 was markedly increased to 57.7 and 71.2% for the 5.05 and 10.1 EU/g of flour BStA dosages, respectively. This increase went hand in hand with a reduction in the level of the larger side chains. The reduction was most pronounced for the side chains with DP 10−11 and 12−16. BStA reduced residual amylopectin CL and β-limit values to a large extent, resulting in CL values of DP 15.7−12.5 and β-limits ranging from 44.5 to 35.3% for the different enzyme dosages. OCL was reduced as well and ranged from DP 9.0 to 6.5 (**Table 5**).

Amylase Action during Breadmaking. Our results clearly show that the different amylases affect the molecular structure of

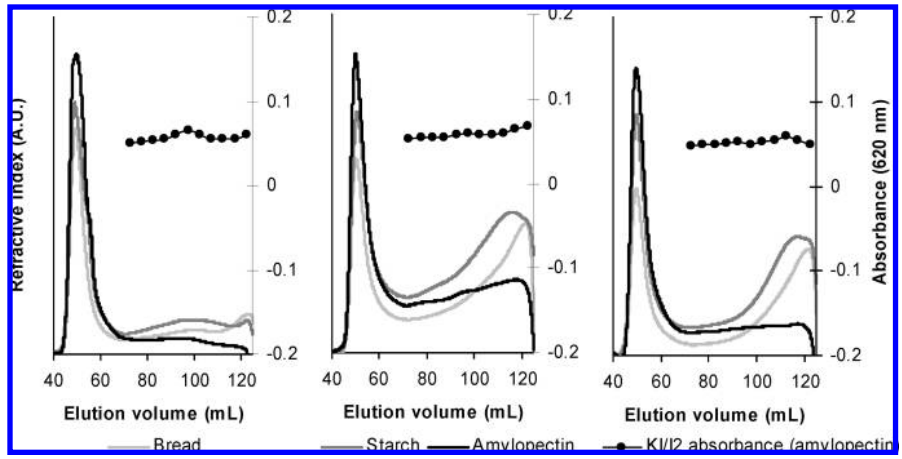

Figure 4. Typical size exclusion chromatography elution profiles of crumb samples and the starch and amylopectin isolated thereof from control (left) and *B. subtilis* α-amylase (0.10 EU/g of flour) (middle) and *B. stearothermophilus* maltogenic amylase (5.05 EU/g of flour) supplemented bread. Dots represent the KI/I_2 absorbance measurements (620 nm) of the eluted fractions obtained after amylopectin analysis.

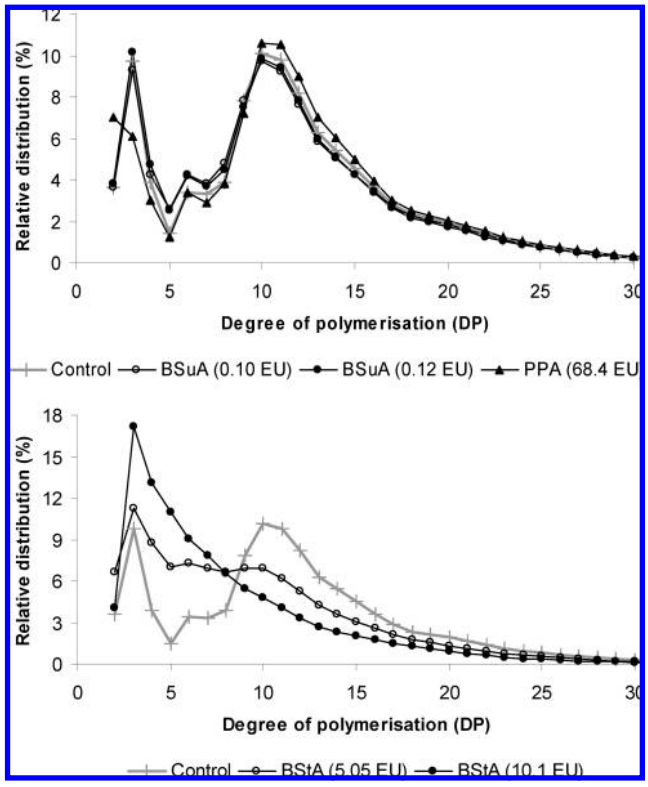

Figure 5. High-performance anion-exchange chromatography analysis of chain length distributions of amylopectin samples. Amylopectin was isolated from control and amylase supplemented bread samples and subsequently debranched with *Pseudomonas* isoamylase. BSuA, *B. subtilis* α-amylase; BStA, *B. stearothermophilus* maltogenic α-amylase; PPA, porcine pancreatic α-amylase.

amylopectin and amylose to different extents and in different ways, related to their thermal stabilities and modes of action. In general, amylase action on the starch polymers during breadmaking is similar to that described earlier for amylase-supplemented starch slurries which were subjected to a heating step (*17*).

Presumably, the endoacting BSuA, at low enzyme activity levels, mainly hydrolyzes the internal bonds in the starch polymers. For amylose, BSuA addition thus reduces amylose MW and its relative content. In the case of amylopectin, it primarily liberates individual clusters or groups of clusters. This leaves the overall amylopectin structure relatively intact, with only relatively small influence in the internal chain distribution of the amylopectin molecules, whereas amylopectin MW can be decreased to a considerable extent.

In general, PPA affected the MW of both amylose and amylopectin and the amylopectin side-chain distribution to a limited extent. Its influence on bread and starch properties was probably more significantly affected by its low thermal stability and inhibition by flour proteins (*21*) than by its multiple attack action (*34*, *35*). Therefore, we will focus less on PPA in the remainder of the discussion.

Table 4. Relative Chain Length Distribution of the Residual Amylopectin after Breadmaking, with and without Amylases Present, Grouped in Different Fractions Based on Their Degree of Polymerization (DP)[a]

amylase (concn, EU/g of flour)	DP ≤ 9	DP 10−11	DP 12−16	DP 17−24	DP ≥ 25
control	35.8 ± 0.7 d	19.2 ± 0.2 a	27.0 ± 0.3 b	13.9 ± 0.1 b	4.1 ± 0.0 b
BSuA (0.10 EU)	39.6 ± 1.5 c	18.5 ± 0.7 a	25.6 ± 0.7 b	12.7 ± 0.2 c	3.6 ± 0.1 c
BSuA (0.12 EU)	38.9 ± 0.6 cd	18.3 ± 0.2 a	25.4 ± 0.2 b	13.3 ± 0.1 bc	4.0 ± 0.0 b
PPA (68.4 EU)	32.3 ± 0.3 e	19.8 ± 0.1 a	29.0 ± 0.1 a	14.5 ± 0.1 a	4.5 ± 0.1 a
BStA (5.05 EU)	57.7 ± 0.7 b	12.4 ± 0.3 b	17.5 ± 0.3 c	9.6 ± 0.1 d	2.8 ± 0.0 d
BStA (10.1 EU)	71.2 ± 0.3 a	8.5 ± 0.1 c	11.7 ± 0.1 d	6.7 ± 0.1 e	2.0 ± 0.1 e

[a] Values followed by different letters in the same column indicate significantly different means at $P < 0.05$. BSuA, *Bacillus subtilis* α-amylase; PPA, porcine pancreatic α-amylase; BStA, *Bacillus stearothermophilus* maltogenic α-amylase.

Table 5. Parameters Describing the Residual Structure of Amylopectin Recovered after Breadmaking with or without Addition of Amylases at Various Concentrations[a]

amylase (concn, EU/g of flour)	CL (DP)	β-limit (%)	OCL (DP)
control	22.4 ± 1.8 a	48.6 ± 2.1 a	12.9
BSuA (0.10 EU)	24.0 ± 0.6 a	46.3 ± 1.4 ab	13.1
BSuA (0.12 EU)	18.6 ± 1.6 ab	45.4 ± 3.3 ab	10.5
BSuA (0.17 EU)	11.1 ± 0.8 d	38.7 ± 2.1 cd	6.3
PPA (68.4 EU)	22.0 ± 0.5 a	44.8 ± 3.4 abc	11.9
BStA (5.05 EU)	15.7 ± 0.7 bc	44.5 ± 3.3 abc	9.0
BStA (10.1 EU)	13.4 ± 1.7 cd	41.4 ± 3.4 cd	7.6
BStA (20.2 EU)	12.5 ± 0.2 d	35.7 ± 0.6 d	6.5

[a] Values followed by different letters in the same column indicate significantly different means at $P < 0.05$. The values of β-limit (β-amylolysis limit, i.e., the molar ratio of reducing ends after incubation with β-amylase to total carbohydrate contents) and CL (average chain length of amylopectin) are reported as mean ± standard deviation; OCL, average outer chain length; BSuA, *Bacillus subtilis* α-amylase; PPA, porcine pancreatic α-amylase; BStA, *Bacillus stearothermophilus* maltogenic α-amylase.

BStA action affected the starch polymers to a large extent. The outer amylopectin chains, more in particular the amylopectin chain segments that are external to the branch points, were extensively degraded. This is in line with its suggested exoaction (*33*, *36*) and is probably even more pronounced due to the addition of rather high enzyme activity levels. In addition, amylose and amylopectin MW were reduced, which indicates that its endoaction during breadmaking cannot be neglected. This combination of a high number of exotype hydrolytic actions and a limited endohydrolysis is in agreement with the reported high to very high levels of multiple attack action (*35*). Furthermore, BStA seems to affect amylose and amylopectin MW more in breadmaking (**Figure 2**) than in the RVA analysis of starch slurries (*17*, *20*). Because BStA has a more pronounced endoaction at higher temperatures (*35*) and survives baking (*22*), this might be explained by the longer time period at higher temperatures in breadmaking than in the RVA analysis. Therefore, we presume that the main endoaction happens primarily at the end of baking (after the crumb has set) and/or during the first stages of cooling.

Antistaling Properties of Amylases. *Bread Staling Model.* As indicated before, both the antistaling mechanism of specific amylases and the staling mechanism itself still are much debated issues. Several hypotheses on crumb firming during storage have been proposed (see, e.g., refs *2* and *3* for an overview). In general, most of them attribute crumb firming primarily to starch retrogradation (see, e.g., refs *2* and *7*). Although our data show a positive correlation between the increase in crumb firmness and the increase in recrystallized amylopectin levels (measured as melting enthalpies in DSC), we believe that amylopectin recrystallization itself cannot explain the bread staling related crumb firming and loss of resilience. In line with the conclusions of Gray and BeMiller (*3*) in their thorough review on bread staling and based on food polymer related concepts (*37*, *38*), we believe that both rearrangements in the starch fraction, network and supermolecular structure formation, and changes in water distribution are involved in crumb quality deterioration.

The softness and resilience of freshly baked (cooled) breads is defined by the different networks occurring in the crumb, particularly the thermoset gluten network and the rather weak partially crystalline amylose network (with crystalline amylose and amylose−lipid complex junction zones). However, the main part of the starch is completely amorphous. During storage, an extensive, partially crystalline, permanent amylopectin network is formed, with junction zones formed by (intermolecular) recrystallization of amylopectin branches. This network further matures during storage, thereby increasing the size and number of both inter- and intramolecular crystalline zones and, hence, contributes to increased crumb firmness. However, amylopectin recrystallization and concomitant network formation during storage also affect water mobility and distribution. Indeed, recrystallized amylopectin consists of B-type crystals, which include 36 water molecules in their crystal unit cell (*39*). Therefore, as amylopectin recrystallization proceeds, more and more water is immobilized in the crystals. This way, the water content of the amorphous starch regions and the gluten phase decreases, as evidenced by the loss of "freezable" water during storage (as measured by DSC) (*38*), and this decrease is even more pronounced due to water migration from crumb to crust (*40*). This "loss" in plasticizing water increases the firmness of the different networks in the bread, particularly the gluten network and the amorphous parts of the starch network, and also decreases the flexibility of the gluten network. The end result of these processes is the perceived drier, firmer, and less resilient texture of stale bread.

Antistaling Action of Amylases. Both the endo-α-amylase BSuA and the maltogenic α-amylase BStA reduced crumb firming during storage. However, both recrystallized amylopectin level (as estimated by the DSC melting enthalpy at d6) as the firmness increase during storage was higher for the BSuA-containing breads than for the BStA samples. In addition, the resilience of the BSuA breads was poor.

As outlined above, BSuA, as the primary example of a conventional endo-α-amylase, mainly hydrolyzed the internal bonds of the starch polymers. Thus, the levels of long chains connecting the different junction zones are reduced, and, concomitantly, the different starch networks are weakened, with a resulting decrease in crumb firmness. This thus concurs with the views of Zobel and Senti (*41*), Senti and Dimler (*42*), and Hug-Iten and co-workers (*7*). Only low enzyme activity levels can be used, because an extensive degradation would result in structural collapse. This risk is particularly relevant because this enzyme survives baking and continues its action during storage. In addition, this enzyme had only little effect on the outer branches of amylopectin (**Figure 5**). Consequently, these branches can still crystallize, which goes hand in hand with incorporation and immobilization of water molecules in the crystal structure. This way, the availability of water, needed for gluten resilience, is decreased, with a resulting decrease in bread quality.

In contrast, the maltogenic BStA mainly depolymerizes the outer amylopectin branches, resulting in a high level of very short amylopectin chains (**Figure 5**). Several studies showed that a high relative amount of very short amylopectin chains (such as DP 6−9 or DP < 11) inhibits amylopectin retrogradation (*43*, *44*). Because at least 10 glucose units are needed for malto-oligosaccharides to form double helices (*45*), BStA action results in many of the outer chains becoming too short to crystallize and form crystalline junction zones. As a consequence, the formation of the permanent amylopectin network during storage is prevented and only the amorphous starch network and the weak amylose network of freshly baked bread are retained. In addition, the crystallization-induced immobilization of plasticizing water is prevented as well, and, thus, the flexibility of the different biopolymer networks, particularly the gluten phase, is retained. The overall effect is a decreased firmness with no significant loss of resilience. In this view, a thermostable β-amylase able to act after the starch is gelatinized would be an effective antistaling enzyme as well. In this context, it is, at present, unclear how the (limited) endoaction observed for BStA contributes to the antifirming mechanism. Possibly, this may result in an additional weakening of the starch networks. In addition, the reduced amylose MW presumably promotes amylose mobility and amylose crystallization and network formation (*7*, *46*). This then leads to a higher initial firmness of the BStA-supplemented breads. In this respect, Hug-Iten and co-workers (*7*) suggested that the enzymically induced fast formation of a starch network contributes to a kinetic texture stabilization, which prevents structure collapse and hinders rearrangements in the starch (amylose) phase, thus contributing to its antifirming effect.

Both BSuA and BStA resulted in a large increase of the hot-water extractable dextrin content in the fresh bread with the average DP depending on the enzyme. High dextrin levels might contribute to the antifirming properties as well. They might hinder the formation of the double helices and/or may act as

antiplasticizers and reduce the mobility of the side chains. However, their impact on starch reassociation seems rather limited, because, particularly in the case of BSuA, despite the high dextrin content, amylopectin recrystallization is reduced to only a limited extent. Furthermore, high dextrin levels may affect crumb relative humidity and thus affect moisture redistribution between crust and crumb.

In conclusion, the antistaling properties of different amylases clearly depend on the properties and action mechanism of the amylases, which determine how amylopectin recrystallization and starch network formation and water distribution and mobility occur during breadmaking and storage. The antistaling properties of a conventional bacterial endo-α-amylase such as BSuA can easily be explained by the enzymically induced reduction in the number of connections between the crystallites in the starch networks. Because the bacterial α-amylase survives the baking phase, these hydrolytic actions continue during storage, which may contribute to its antifirming effect, but also increases the risks of applying an overdose. However, because the enzyme has only little impact on the outer amylopectin chains, amylopectin recrystallization is not hindered, resulting in decreased levels of plasticizing water and poor crumb resilience. In breadmaking, the maltogenic amylase BStA acts as an exoacting amylase with more pronounced endoaction at higher temperatures. The antifirming mechanism of BStA can primarily be ascribed to the extensive degradation of the crystallizable amylopectin side chains, which limits the formation of a permanent amylopectin network during storage and prevents the incorporation of the plasticizing water in the amylopectin crystallites. This way, the different starch and gluten networks keep their flexibility, resulting in a soft crumb with good resilience. In addition, the formation of high levels of dextrins, particularly in the case of BStA (mainly maltose), may contribute to the antifirming properties as well. Finally, although PPA with its multiple attack actions can be considered as exhibiting both endo- and exotypes of actions, its low thermal stability and inhibition by flour proteins probably explains the limited effect of PPA on bread and starch properties compared to the control.

ABBREVIATIONS USED

BStA, *Bacillus stearothermophilus* maltogenic α-amylase; BSuA, *Bacillus subtilis* endo-α-amylase; CL, average chain length; DP, degree of polymerization; MW, molecular weight; OCL, average outer chain length; PPA, porcine pancreatic α-amylase; RVA, rapid visco analyzer; SEC, size exclusion chromatography.

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