



# Determination of the Direct Activity of the Maltogenic Amylase from *Geobacillus stearothermophilus* in White Bread

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## Abstract

An assay-based method was developed to determine the residual activity of the maltogenic amylase from *Geobacillus stearothermophilus* in white bread. It was found that the important step for amylase extraction from the bread matrix was the addition of 10% (w/v) maltodextrin in the extraction buffer. The endogenous amylase activity in dough was investigated, and its inactivation during bread baking was proven. Thus, all amylase activities measured after baking have an exogenous origin. The amylase activities in the loaf of self-baked white bread containing defined dosages of exogenous amylase (10–100 µg per g flour) were reproducibly determined with  $17.8 \pm 1.24\%$  residual activity. Moreover, an amylase activity of  $369 \pm 34.3 \text{ pkat g}_{\text{bread}}^{-1}$  was determined in three batches of a commercial white bread. The real temperature impact on the amylase during bread baking was investigated. The highest temperature in the crumb was 97 °C and, therefore, is significantly lower than the oven temperature (230 °C).

**Keywords** Maltogenic amylase · Thermal stability · Residual activity · Bread baking · Enzyme declaration · Heat inactivation

## Introduction

Amylases of bacterial and fungal origin have been used in the baking industry for several decades without labeling them on the baked goods. They are used to influence bread color and flavor and to retard staling (Lagrain et al. 2008; de Souza and de Oliveira 2010). One example of an amylase which is commercially used is the maltogenic amylase from *Geobacillus stearothermophilus* (formerly known as *Bacillus stearothermophilus*), which can be purchased under the trade name Novamyl (Novozyme). Maltogenic amylases (EC 3.2.1.133, glucan-1,4- $\alpha$ -maltohydrolase) hydrolyze starch and remove successive  $\alpha$ -maltose from the non-reducing end of the polysaccharide chain (Christophersen et al. 1998).

Enzymes added to food can be seen as processing aids if they have no technological function in the final product. This is the

case if they are inactivated completely during food processing or if a residual enzyme activity shows no technical function in the final product. According to the regulation (EG) Nr.1332/2008, processing aids do not have to be labeled (The European Parliament and the Council of the European Union 2008). Amylase preparations are used in the baking industry without labeling, since up to now a technical function of the amylase preparation in the final product was not proven. However, there are preliminary studies that some amylases are not inactivated completely during the baking process. Lagrain et al. (2008) investigated the influence of thermostable amylases on the bread crumb structure and texture and observed incomplete inactivation of the maltogenic amylase from *G. stearothermophilus* after baking. This was assumed by determining the degree of starch polymerization over the baking process. The degree of polymerization decreased even after the heating step. As a result, the amylase did not seem to be fully inactivated (Lagrain et al. 2008). The same observation was found for the  $\alpha$ -amylase from *Bacillus subtilis* (Goesaert et al. 2009). Until now, there has been no direct assay-based measurement of any amylase activity in bread described in the literature. The aim of this study was to develop such a method. White bread and the maltogenic amylase from *G. stearothermophilus* were taken as a model system. Finally, commercially available white bread (“toast bread”) was analyzed for quantitative maltogenic amylase activity.

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## Material and Methods

### Chemicals and Materials

All chemicals were of analytical grade and obtained from Sigma-Aldrich (Taufkirchen, Germany), Carl Roth GmbH (Karlsruhe, Germany), AppliChem (Darmstadt, Germany), and Serva (Heidelberg, Germany). Exo-amylase assay reagent (Betamyl3 reagent) was purchased from Megazyme International Ireland (Wicklow, Ireland; lot number: 180301, expiry year: 2023). Different batches of commercial white bread (“toast bread”)—Golden Toast, Buttertoast (expiry dates and labels: 17.03.2019 (SC1) (batch 1), 22.03.2019 (SD7) (batch 2), and 26.03.2019 (SD4) (batch 3))—were bought in a local supermarket. All batches tested were measured before the expiration date.

Wheat flour type 550 was purchased from Rettenmeier Mühle (batch number: 70001073, best before 16.10.2019). Commercial dry yeast was purchased from Saf-instant (LeSaffre, Bühl, Germany) and hydrogenated peanut fat from BÄKO (Ladenburg, Germany). Sourdough starter (Böcker TK Starter, article number: 82001600, batch number: 10048950, expiry date: 27.08.2019) was purchased from Ernst Böcker GmbH (Minden, Germany). All buffers and solutions were prepared using double-distilled and autoclaved water. All reaction tubes, glass vessels, spoons, and other similar objects were autoclaved before use to minimize the risk of amylase contamination. All working steps were conducted wearing gloves.

### Statistics

All baking experiments, extractions, and activity determinations were carried out in triplicate. Standard deviations were calculated using Excel (Microsoft, Redmond, USA). The limit of detection (LOD) and the limit of quantification (LOQ) were evaluated according to DIN ISO 5725-2 and DIN 32646, respectively (Gey 2008). Thus, the LOD of the assay is defined as six times the standard deviation of the blank values. The LOQ is defined by nine times the standard deviation of the blank values.

### Amylase Preparation Used

A powdered food-grade and food-approved amylase preparation of the maltogenic amylase from *G. stearothermophilus* was used for the baking experiments. White bread was made with three different enzyme dosages covering the recommendation from the manufacturer (10–100 µg per g flour, given by the manufacturer in parts per million (ppm) regarding flour mass). The amylase was added at 10, 50, and 100 µg per g flour. In order to determine the specific activity of the amylase preparation used, the powder was suspended in standard assay buffer (50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 2 mM CaCl<sub>2</sub>, 0.1 % (w/v) Na-azide, pH 5.5) with 1.5 g bovine serum

albumin (BSA) per liter. The suspension was prepared with an amylase preparation concentration of 15 g per liter and shaken for 45 min at 20 °C. Then, the suspension was centrifuged at 8000 rpm for 10 min at 4 °C. The supernatant was loaded on PD10 columns and eluted using 50 mM MES, 2 mM CaCl<sub>2</sub>, and 0.1% (w/v) Na-azide, pH 5.5. The final concentration of the amylase preparation was 10 g L<sup>-1</sup> with a concentration of 1.0 g BSA per liter due to the dilution during the buffer change. This extraction procedure was carried out in triplicate, and the enzyme solutions were used to determine the activity. The average of the three activity values was used for the calculation of the percentage residual activity of the baking experiments.

### Assay for Determining the Exo-Amylase Activity

The maltogenic amylase from *G. stearothermophilus* was investigated using the exo-amylase Betamyl3 reagent from Megazyme (Wicklow, Ireland). This reagent contained β-glucosidase and *p*-nitrophenyl-β-D-maltotriose (PNPβ-G3). Amylases with an exo-acting activity can cleave the *p*-nitrophenyl-β-D-maltotriose to maltose and *p*-nitrophenyl-β-D-glucose. The *p*-nitrophenyl-β-D-glucose is immediately cleaved to D-glucose and *p*-nitrophenol by β-glucosidase. The activity assay was carried out on a small scale. Therefore, 50 µL preincubated bread or dough extract (3 min, 40 °C) (see the “Optimization of the Extraction Procedure” section) was mixed with 50 µL preincubated Betamyl3 reagent (3 min, 40 °C). Once the solution had turned yellow, the reaction was stopped with 375 µL of 2% (w/v) Tris, pH 11.0. A quantity of 240 µL was transferred to a microtiter plate and measured at 405 nm. A calibration curve with *p*-nitrophenol with concentrations between 0.003 and 0.2 mM was made. Two different blanks (B) were tested for the evaluation of the assay. For B1, which was recommended for an assay incubation time of 10 min by the manufacturer, the amylase sample was added after incubation and stopping of the assay solution. For B2, Betamyl3 reagent was incubated with standard assay buffer (50 mM MES, 2 mM CaCl<sub>2</sub>, 1 g L<sup>-1</sup> BSA, 0.1% (w/v) Na-azide) during the assay run time, followed by the addition of the stopping reagent. Nine B2 were made for an assay run time of 24 h for the determination of the minimal detectable (LOD) and quantifiable (LOQ) volumetric activity. A solution of the maltogenic amylase from *G. stearothermophilus* in standard assay buffer was diluted to volumetric activities between 0.5 and 100.0 pkat mL<sup>-1</sup> to check whether long assay run times lead to reliable results.

### Production of White Bread, Sample Taking, and Measurement of the Temperature Profile During Baking

The self-baked white bread consists of wheat flour type 550 (450 g with regard to 14% relative moisture content), dry yeast

(4.5 g), sodium chloride (6.75 g), sucrose (4.5 g), fat (4.5 g), and water (300 g). The maltogenic amylase was added together with the water to the dough ingredients. The ingredients were mixed in a spiral mixer (Hobart kneader) for 2 min at level 1 and 5 min at level 2. The primary fermentation step was carried out for 20 min at 31 °C with 75 to 85% relative humidity in a proofing cabinet. The next step was a dough relaxation phase of 10 min at room temperature. The dough was shaped, placed in the baking pan, and proofed for 60 min at 31 °C and 75 to 85% relative humidity in a proofing cabinet. The baking was performed at 230 °C for 35 min with 8 s vapor. A white bread loaf was produced with sourdough (Böcker TK starter) for an estimation of the relevance of the latter on the determination of residual amylase activity in commercial white bread. The sourdough white bread loaf contained the same ingredients as the other white bread loaves. Part of the wheat flour and water was used for the preparation of a sourdough starter.

A quantity of 13.5 g of the frozen sourdough was mixed with part of the wheat flour and water for one white loaf. The ingredients were mixed, and the sourdough starter was incubated for 17 h at 26 °C. Then, the remaining wheat flour, water, and the other ingredients were added and mixed. Further steps were carried as described previously.

The temperature profile of the baking step was recorded using a digital thermometer (Almemo 2590, Ahlborn). The slide wires were stuck into the bread halfway up its height. The temperature was recorded every 30 s, and the measurement was carried out in triplicate. After baking, the bread was removed from the pan and cooled at room temperature. Samples for determining the activity were taken directly after mixing, before baking, and 2 h after baking. Slices of bread taken from the middle part of the loaf were used for the samples. Only the bread crumb was used to measure the activity. All calculated values were referred to the bread loaf. Sample taking from the bread slices is shown in Fig. 4 in the Appendix.

Samples were directly frozen at  $-80$  °C. White bread loaves were made with three different enzyme dosages. Bread without enzyme supplementation was used as a reference. Samples from commercial white bread were taken in the same manner.

### Optimization of the Extraction Procedure

The dough and bread samples were freeze-dried and ground to extract the maltogenic amylase. Samples were weighed before and after drying to calculate a drying factor to relate the exo-amylase activities determined to 1 g dough or bread. The dried samples were ground using a conical drill. Extractions were carried out with one-part freeze-dried sample (0.17 g) and ten parts (1.7 mL) of the respective buffer in autoclaved reaction tubes (2 mL). After the buffer was added, the samples were

vortexed carefully and then shaken at 100 rpm at 20 °C on an orbital shaker for a defined time. The samples were centrifuged at 20,000g for 20 min at 4 °C. The supernatant was loaded on a PD-10 desalting column to change the buffer to standard assay buffer (50 mM MES, 2 mM  $\text{CaCl}_2$ , 1 g  $\text{L}^{-1}$  BSA, 0.1% (w/v) Na-azide, pH 5.5). Standard extraction buffer MES (50 mM), with  $\text{CaCl}_2$  (2 mM), BSA (1.5 g  $\text{L}^{-1}$ ), and Na-azide (0.1% (w/v)), at pH 5.5 and an extraction time of 1 h was used as a starting point. Maltose (1 M) and maltodextrin (10 and 20% (w/v), respectively) were tested as extraction additives. The buffer extraction time was varied between 1 and 5 h to find the best one. The best conditions were used for later exo-amylase determinations. The activity of a self-baked white bread (100  $\mu\text{g}$  per g flour) was determined independently on five consecutive days using new solutions every day to determine the repeatability of the extraction procedure. Each sample was freeze-dried on the day before determining the activity.

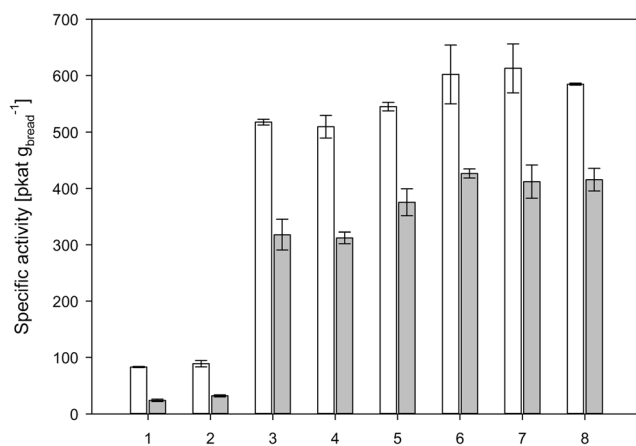
## Results and Discussion

### Finding the Reliable Blank and Run Time for the Assay

The original protocol of the Betamyl3 assay was used as a starting point to develop a reliable assay for the determination of maltogenic amylase activity. An assay run time of 10 min was usually recommended (Megazyme 2018). The sensitivity of this assay had to be improved by prolonging the assay run time to ensure the detection of very low amylase activities in the white bread. Solutions of the maltogenic amylase from *Geobacillus stearothermophilus* with specified activities between 0.5 and 100 pkat  $\text{mL}^{-1}$  were used to challenge the assay for the evaluation of longer assay run times. A suitable blank (zero value of enzymatic reaction) also had to be defined, since the assay was based on the difference of absorption measurements. In addition to the recommended blank, where the amylase sample was added after incubation and stopping of the assay solution (B1), our blank added the heat-inactivated amylase sample or buffer, respectively, before incubation and stopping (B2) (see the “Material and Methods” section). The use of a heat-inactivated sample and buffer led to the same absorption value for B2. Thus, only buffer was used for B2. In further experiments, the comparison of both blanks demonstrated that B2 was reliable in determining 1 pkat exo-amylase activity per milliliter within an assay run time of 24 h (Table 3 in the Appendix). The limit of detection (LOD) and limit of quantification (LOQ) for an assay run time of 24 h were 0.6 and 0.9 pkat  $\text{mL}^{-1}$ , respectively. In summary, these modifications resulted in a reliable assay suitable to investigate very low maltogenic amylase activities.

## Development of an Extraction Method for Amylase from White Bread

A suitable extraction method for the determination of amylase activity in white bread had to be developed. Pretests showed that there is a high risk of an amylase contamination from the laboratory environment. Therefore, only amylase-free materials were used (see the “Material and Methods” section). A freeze-drying step was included for convenient handling of different white bread samples which equalized the moisture content of the samples. Furthermore, freeze-drying of the samples allowed the secure handling of high sample numbers as freeze-dried white bread was ground and stored in small reaction vessels. The freeze-drying of the samples had no inactivation effect on the amylase. Freeze-dried samples were stored for up to three days without loss of amylase activity (data not shown). Thus, the further improvement of the extraction method was carried out with freeze-dried bread samples. The method was developed by using self-baked white bread as a matrix and adding known amylase activities to the dough. The self-baked white bread was made with 100  $\mu\text{g}$  of the maltogenic amylase from *G. stearothermophilus* per g flour, which corresponds to the upper limit of the dosage recommendation. In the beginning, only a low amylase activity was determined in the self-baked white bread when using pure buffer solution for extraction (Fig. 1, bar 1). One reason for the low amylase activity in the buffer could have been an insufficient extraction of the amylase from the starch molecules in the bread sample. It was described in the literature that the binding of amylases to starch is reversible, as reported for amylase purification, by using starch columns. Here, the amylase was detached from starch when particular additives, such as maltose or maltodextrin, were present in the eluent solution (Subbaramaiah and Sharma 1985; Weber et al.



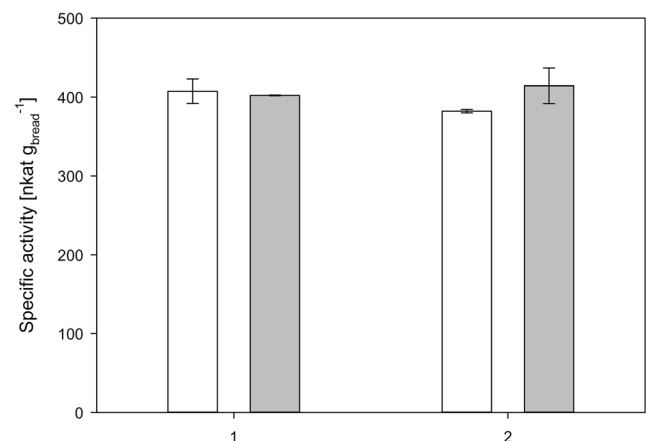
**Fig. 1.** Extraction of amylase activities from self-baked (white bars) and commercially available white bread (gray bars) using additives in the extraction buffer (bar 1, extraction buffer without additives; bar 2, plus 1 M maltose; bar 3, plus 20% (w/v) maltodextrin; bars 4–8, plus 10% (w/v) maltodextrin) and different extraction times (bars 1–4, incubation 1 h; bars 5–8, incubation at 2, 3, 4, and 5 h, respectively)

1976). Therefore, these additives were tested for amylase extraction from the self-baked and commercially available white bread (Fig. 1, bars 2–4).

As Fig. 2 shows, the addition of 10% (w/v) maltodextrin resulted in a 6-fold increase of the amylase activity in the case of self-baked white bread and a 13-fold increase in the case of commercially available white bread compared to pure extraction buffer. The prolongation of the extraction time from 1 to 3 h resulted in a further activity increase of 18% (self-baked white bread) and 34% (commercially available white bread). Further prolongation of the extraction time did not lead to higher amylase activities (Fig. 1). Thus, the following extraction experiments were carried out using 10% (w/v) maltodextrin in the extraction buffer and an extraction time of 3 h. Furthermore, the principle to get a better extraction of amylases from bread samples using a specific additive in the extraction buffer could also be used for other amylases. In our experience, the additives had to be investigated empirically for the individual amylase.

## Amylase Activities in White Bread Production

White bread loaves were produced with defined dosages of the maltogenic amylase from *G. stearothermophilus* to investigate the exogenous amylase activities before and after baking. The fact that the dough also contained endogenous amylase activities had to be considered. These activities originated from the wheat flour and yeast. Therefore, firstly, a reference dough without the addition of exogenous amylase was prepared and its endogenous amylase activity before and after the rising of the dough was checked. This resulted in  $407.3 \pm 15.4$  and  $382.1 \pm 2.1$  nkat g<sub>dough</sub><sup>-1</sup>, respectively, which is no significant difference (data shown in Fig. 2). The difference between these two values is 6.6% and indicates the natural variation of the developed extraction method. The exogenous



**Fig. 2.** Amylase activities in the dough of white bread with 100  $\mu\text{g}$  maltogenic amylase per g wheat flour (gray bars) and in the reference bread (white bars). 1, sample directly after kneading; 2, sample before baking



amylase from *G. stearothermophilus* was added to the dough up to a maximum of 100 µg per g flour in the following experiments. This corresponded theoretically to an amylase activity of 3.4 nkat g<sub>dough</sub><sup>-1</sup>. In conclusion, the endogenous amylase activities in dough were more than 100-fold higher than the added exogenous *G. stearothermophilus* amylase activity and cannot be distinguished in the dough. The reference dough without added exogenous amylase was baked (230 °C, 35 min), and the resulting white bread was termed reference bread. Amylase activity was not detected in this reference bread (see Table 1). Thus, any amylase activities measured in the self-baked white bread in further experiments must originate from exogenous amylases which were added to the dough.

The main difference between endogenous wheat flour or yeast amylases and the exogenous amylase from *G. stearothermophilus* was the extraordinary heat stability of the latter amylase, with a temperature optimum between 60 and 65 °C (Derde et al. 2012).

### Residual Exogenous Amylase Activities in Baked White Bread

Self-baked white bread loaves were prepared by using different dosages of the maltogenic amylase from *G. stearothermophilus* within the range of the manufacturer's recommendation (10–100 µg per g flour). Samples were taken from the self-baked bread, which were then freeze-dried, ground, and extracted using the extraction method developed (see the “Development of an Extraction Method for Amylase from White Bread” section). The amylase activities of the extracts were then determined (Table 1).

The residual amylase activities of the loaves prepared with exogenous amylase after baking were between 71.2 ± 2.54 and 733.9 ± 40.35 pkat g<sub>bread</sub><sup>-1</sup>. The residual amylase activity was at an average of 17.8 ± 1.24% among the dosages tested. As mentioned previously, the reference bread (RB) without added amylase did not show any residual amylase activity. For the first time, amylase activity was directly measured in

bread using an activity-based method and it was possible to distinguish clearly between endogenous and exogenous amylase activities in the final bread because the endogenous amylases were inactivated by the baking process. To evaluate the repeatability of the extraction and assay methods according to ICH (1994), five amylase samples from one white bread loaf baked with 100 µg maltogenic amylase per g flour were taken on the day of baking and stored at - 80 °C. The amylase activity of these samples was determined independently each per day over five days after freeze-drying of each sample overnight and extraction of the amylase as described previously, using new solutions for each sample (Table 4 in the Appendix). An amylase activity of an average of about 786.9 ± 28.3 pkat g<sub>bread</sub><sup>-1</sup> was measured for the samples derived from a single bread. This is in accordance with the data for the bread loaf with 100 µg maltogenic amylase per g flour which were determined in the former experiments from three different bread loaves (733.9 ± 40.35 pkat g<sub>bread</sub><sup>-1</sup>; see Table 1). The variation of 3.6% is in a similar range as the variation of the dough samples (6.6%).

Two studies were found in the literature which also reported activities of the maltogenic amylase from *G. stearothermophilus* and an α-amylase from *Bacillus subtilis* in bread baking (Goesaert et al. 2009; Lagrain et al. 2008). Both studies used indirect methods by either investigating the molecular weight distribution of starch in the baked bread (Lagrain et al. 2008) or analyzing the hot-water extractable dextrins from baked bread (Goesaert et al. 2009). In both studies, the composition of the starch molecules changed after the bread baking step and, thus, suggested an incomplete inactivation of the amylases tested. These results are in accordance with the results of our study. The studies of Lagrain et al. (2008) and Goesaert et al. (2009) gave no information about the true inactivation of the amylases during the heating step in bread baking. In our study, it was possible for the first time to estimate the inactivation of the maltogenic amylase from *G. stearothermophilus* during bread baking. Using the bread crumb, the inactivation was determined at about 82%, although the dosage of amylase varied from 10 to 100 µg per g flour in the bread (see Table 1).

**Table 1.** Amylase activities in white bread after baking using different dosages of the maltogenic amylase from *Geobacillus stearothermophilus*

Dosage (µg per g flour)	Added amylase activity (pkat g <sub>bread</sub> <sup>-1</sup> )	Amylase activity after baking	
		(pkat g <sub>bread</sub> <sup>-1</sup> ) <sup>b</sup>	(%)
0 (RB) <sup>a</sup>	0	0	-
10	387.4	71.2 ± 2.54	18.4 ± 0.66
50	1943.6	311.5 ± 29.81	16.0 ± 1.5
100	3884.8	733.9 ± 40.35	18.9 ± 1.0

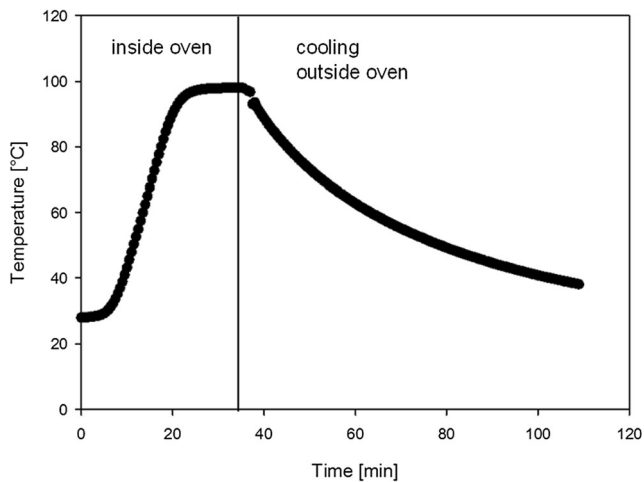
<sup>a</sup> RB, reference bread (without addition of exogenous amylase)

<sup>b</sup> LOD ≤ 2.4 pkat g<sub>bread</sub><sup>-1</sup>

### Temperature Impact During Baking

The temperature inside the bread crumb was recorded over the baking and cooling processes to investigate the true temperature course that acts on the amylase (Fig. 3).

The temperature in the bread crumb reached a maximal value of around 97 °C after 20 min. This temperature was held for about 17 min. The temperature profile of the baking and cooling of white bread was also recorded by Lagrain et al. (2008). Both temperature courses inside the bread showed similar stages despite variations in bread mass (740 g instead of 450 g), baking time, and temperature (40 min at 210 °C instead of 35 min at



**Fig. 3.** Temperature profile of the baking and cooling processes in the middle of the white bread (3–4 cm from the crust)

230 °C). The temperature in the study of Lagrain et al. (2008) reached the maximal value of about 97 °C after 27 min. This value was held for about 20 min. It was assumed that the temperature impact on the maltogenic amylase in our study could be regarded as representative of the baking of white bread due to the similarity of the temperature profiles. The general thermal stability of the maltogenic amylase from *G. stearothermophilus* is well-known. Derde et al. (2012) investigated its thermal stability in buffer. They incubated the maltogenic amylase in sodium maleate buffer (100 mM) with  $\text{CaCl}_2$  (5 mM) at pH 6.0 for 30 min at temperatures between 20 and 90 °C. The residual activity decreased with increasing temperature. Although 50% of the initial activity was still present at 80 °C, the amylase was completely inactivated at 90 °C (Derde et al. 2012). The thermal stability of the maltogenic amylase from *G. stearothermophilus* in buffer may also have contributed to the assumption that this amylase is completely inactivated during bread baking. The recent study suggested a stabilizing effect of the bread matrix on the maltogenic amylase in dough/bread, since about 18% of the initial activity was still present in this study after 20 min at temperatures above 90 °C. A stabilizing effect in the dough/bread could originate from the presence of the substrate starch and/or from the low water activity. Such a matrix effect was reported by Mamo et al. (1999). They investigated the thermal stability of an amylase from *Bacillus* sp. WN11 in the presence of starch at concentrations from 5 to 20% starch at 100 °C. Thereby, the residual activity after 5-min incubation increased with increasing starch concentrations from 25 to 85% (Mamo et al. 1999). The reduction of the free water activity ( $a_w$  values) also led to an enhanced thermal stability. Terebiznik et al. (1997) observed a great increase in the thermal stability of the  $\alpha$ -amylase from *Aspergillus oryzae* NRRL 3485 by reduction of the water content (Terebiznik et al. 1997). The phenomenon of enhanced thermal stability in the presence of low moisture content or the enzyme substrate is widely known. One reason for enhanced thermal stability in a low water content is the assumption

that the freedom of movement of the protein molecules (peptide chains) decreases and, therefore, conformational changes, which could lead to activity losses, are hindered (Terebiznik et al. 1997). These stabilizing effects most probably contributed to the enhanced thermal stability of the maltogenic amylase from *G. stearothermophilus* during bread baking and, thus, might also be true for several other thermostable enzyme preparations used in the baking industry.

### Determination of the Residual Activity in Commercial White Bread

The fact that sourdough was used (declared by producer) for the production of the commercially available white bread tested had to be considered when investigating the residual activity in the latter. Thus, a self-baked white bread loaf containing sourdough without added amylase was prepared to investigate the relevance of sourdough in white bread as a potential ingredient containing amylase. However, the bread with sourdough also showed no activity. Thus, it was proven that the addition of sourdough did not lead to a residual amylase activity in the commercially available white bread tested. Commercially available white bread loaves were bought in a local supermarket. Three different batches of a commercially available white bread were tested for residual amylase activity. The activity values determined are shown in Table 2.

The average amylase activity of the three batches was  $369 \pm 34.3$  pkat  $\text{g}_{\text{bread}}^{-1}$ . Therefore, the commercially available white bread loaves analyzed had a relatively constant level of amylase activity. Although it is not known whether the commercially available white bread contained an exogenous amylase preparation, an assumption of the dosage of a possibly added maltogenic amylase can be calculated. Based on the activity measured in Table 2, a dosage of about 50  $\mu\text{g}$  per g flour equivalent to maltogenic amylase can be suspected (corresponding to an activity of  $311.5 \pm 29.81$  pkat  $\text{g}_{\text{bread}}^{-1}$  in Table 1). This assumption fits perfectly in the preparation of a typical white bread loaf.

**Table 2.** Amylase activities determined in commercially available white bread loaves

Batch		Amylase activities from different breads from one batch (pkat $\text{g}_{\text{bread}}^{-1}$ )	Average amylase activity from one batch (pkat $\text{g}_{\text{bread}}^{-1}$ )
1	a	$353.6 \pm 6.4$	$331.4 \pm 15.9$
	b	$321.9 \pm 17.2$	
	c	$318.6 \pm 19.0$	
2	a	$402.3 \pm 2.2$	$414.2 \pm 7.9$
	b	$415.9 \pm 7.6$	
	c	$420.6 \pm 14.3$	
3	a	$322.2 \pm 8.8$	$360.2 \pm 21.3$
	b	$367.1 \pm 8.7$	
	c	$365.5 \pm 8.5$	

## Conclusion

A reliable method for the determination of residual activities from the maltogenic amylase of *G. stearothermophilus* in white bread was developed. The exogenous maltogenic amylase withstood the baking process of bread, and it was shown that there was no endogenous amylase activity from wheat flour or yeast determinable after bread baking. Furthermore, it was proven that commercially available white bread showed a residual amylase activity in a similar range compared to our self-baked white bread loaves.

This study could help in the discussion on labeling enzymes on the bread package. However, a residual amylase activity in bread does not lead to an obligatory declaration. Enzymes just have to be labeled if they have a technological function in the final product. Further efforts have to be made to show a direct correlation of residual amylase activity and a technological function in the white bread. It could be that the remaining maltogenic amylase is contributing to a retardation of staling. Finally, the thermal stability of other amylases used in the baking industry should be investigated to evaluate whether other commercially available enzyme preparations withstand the baking process and are active in the final product.

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## Compliance with Ethical Standards

**Conflict of Interest** Katrin Reichenberger declares that she has no conflict of interest. Anja Luz declares that she has no conflict of interest. Ines Seidl declares that she has no conflict of interest. Lutz Fischer declares that he has no conflict of interest.

**Ethical Approval** This study does not contain experiments that involve human or animals other than the authors who performed the work.

**Informed Consent** All authors have read the manuscript and agreed upon its submission to the journal.

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