TECHNISCHE UNIVERSITÄT MÜNCHEN

TUM School of Life Sciences

Elucidation of the impact of exogenous amylase activity on wheat bread making

Gerold Felix Rebholz

Vollständiger Abdruck der von der TUM School of Life Sciences der Technischen Universität München zur Erlangung des akademischen Grades des

Doktors der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

Vorsitzender:	Prof. Dr. Michael Rychlik
Prüfer der Dissertation:	1. Prof. Dr. Katharina Scherf
	2. Prof. Dr. Wilfried Schwab

Die Dissertation wurde am 06.04.2021 bei der Technischen Universität München eingereicht und durch die TUM School of Life Sciences am 22.06.2021 angenommen.

Danksagung

Zuallererst möchte ich mich bei der Betreuerin meiner Arbeit, Prof. Dr. Katharina Scherf bedanken. Vielen Dank, Kathi, für die Überlassung des Themas, die konstruktiven Gespräche und Diskussionen, die hervorragende Zusammenarbeit sowie den Raum für eigene Ideen.

Ich möchte mich bei allen Kolleginnen und Kollegen des Leibniz-LSB, für die sehr gute Arbeitsatmosphäre und die stetige Hilfsbereitschaft und diverse Ein-, Ausstands- und Triathlon-Feiern bedanken.

Danke auch an die Kolleginnen und Kollegen vom Lehrstuhl für Lebensmittelchemie und Molekulare Sensorik für die Institutsübergreifende unkomplizierte Zusammenarbeit. Ein großes Dankeschön an Karin Sebald für die Unterstützung bei den TOF Messungen, Verena Mittermeier für allerlei Proteomics-Fachsimpelei.

Weiter gilt mein Dank Mario Jekle und Thekla Alpers vom Lehrstuhl für Brau- und Getränketechnologie für die unkomplizierte und sehr gute Zusammenarbeit.

Ein großes Dankeschön an den AK Getreide für das sehr gute Arbeitsklima, die sehr große Hilfsbereitschaft, konstruktive und weniger konstruktive Diskussionen, lustige Gespräche und AK-Feiern.

Vielen Dank dem Labor O.52 für die konstruktive und witzige Zeit am Institut Ein besonders großes Dankeschön an Barbara Lexhaller, Sabrina Geißlitz und Marie Lay für euer großes Interesse an meinem Projekt, die vielen sehr guten Ideen und neuen Perspektiven sowie der Begeisterung fürs Feierabendbier.

Ein großes Dankeschön auch ans Mensa-Team, an Annika Schulz, Benne Bächler, Barbara Lexhaller, Richard Hammerl, Karin Sebald, Philip Pirkwieser, Marie Lay und Sabrina Geißlitz für die fast tägliche Wallfahrt in die Mensa und die guten Gespräche.

Dankeschön an Steffi Hackl und Angelika Grassl für die sehr große Unterstützung meiner Praktischen Arbeit, die Geduld und die Flexibilität gegenüber meiner Versuchsplanung.

Ein ganz besonderes Dankeschön an das Laufteam Lebensmittelchemie, an Alin Kraus, Bene Bächler und Daniela Füllemann, für die vielen Runden durch den Weltwald, die Begeisterung für den Weihenstephaner Berglauf, die Ablenkung von Institut und Promotion und euer offenes Ohr für sämtliche Themen.

Danke Valli für die Fachsimpelei über Enzyme, deine Methodischen Ideen, aber vor allem die Gespräche über Essen und Trinken sowie über sämtliche anderen Themen.

Der größte Dank geht an alle die mich abseits des Institutes unterstützt, motiviert und abgelenkt haben. Danke an Liane und Gerold, Anna und Andreas, Irmgard, Harald und natürlich Leonie!

Vorbemerkung

Der praktische Teil der vorliegenden Arbeit wurde von Juli 2017 bis Juli 2020 unter Leitung von Prof. Dr. Katharina Scherf am Leibniz-Institut für Lebensmittel-Systembiologie an der Technischen Universität München in Freising durchgeführt.

Publikationen

Teile dieser Arbeit wurden vorab in international anerkannten Fachzeitschriften veröffentlicht.

Rebholz GF, Sebald K, Dirndorfer S, Dawid C, Hofmann T, Scherf KA (2021) European Food Research and Technology, DOI: 10.1007/s00217-020-03657-y. Impact of exogenous α-amylases on sugar formation in straight dough wheat bread

Rebholz GF, Sebald K, Dirndorfer S, Dawid C, Hofmann T, Scherf KA (2021) European Food Research and Technology, DOI: 10.1007/s00217-021-03721-1. Impact of exogenous maltogenic α-amylases and maltotetragenic amylase on sugar release in wheat bread

Table of contents

Abbreviations

Cas9	CRISPR associated protein 9
CAZy	Carbohydrate Active Enzymes nomenclature
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
DDA	Data dependent acquisition
DIA	Data independent acquisition
EFSA	European Food Safety Authority
ESI	Electrospray ionization
GC	Gas chromatography
GMO	Genetically modified organism
HPAEC	High-performance anion exchange chromatography
HPLC	High-performance liquid chromatography
iBAQ	Intensity based absolute quantitation
IUBMB	International Union of Biochemistry and Molecular Biology
kat	Katal
LC	Liquid chromatography
LMA	Late maturity α-amylase
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
M_r	Relative molecular mass
nkat	Nanokatal
PAD	Pulsed amperometric detection
PHS	Preharvest sprouting
pI	Isoelectric point
QQQ	Triple quadrupole
SPE	Solid phase extraction
TOF	Time-of-flight

1. Introduction

1.1 Enzymes in wheat bread making

1.1.1 Overview of enzymes applied in wheat bread making

Enzymes have great influence on the quality of wheat bread. Some enzymes are endogenously present in wheat, but their content in the grain kernel is strongly dependent on the environmental conditions during growth, such as temperature, water availability and fertilization [1]. Furthermore, the content of endogenous enzymes usually is too low to have a technological impact on dough or bread characteristics [2, 3]. Thus, various types of exogenous enzymes, especially amylases, xylanases, lipases and carbohydrate oxidases are used in wheat bread making [2, 4].

Amylases are the most frequently applied exogenous enzymes in bakeries [3]. By degrading starch into smaller fragments, they have an impact on the molecular structure of the starch and on the amount of mono-, di- and oligosaccharides in wheat dough and bread [2]. Different types of amylase have different technological effects on dough and bread and are used in wheat bread baking. The most important exogenous ones are α-amylase (EC 3.2.1.1), maltogenic α-amylase (EC 3.2.1.133) and maltotetraogenic amylase (EC 3.2.1.60). Chapter 1.2.3 gives a detailed overview about the different exogenous amylases and their technological impact in wheat bread making.

Xylanases or endo-β-1,4-xylanases are enzymes of the EC 3.2.1.8 group which hydrolyze 1,4-β-D-xylosidic linkages in xylans. Based on genetic information and structural properties, they are mainly grouped into the families 10 and 11 of the glycosyl hydrolase family [5]. The structure of a family 10 xylanase is a 8-fold β/α-barrel, whereas the active site is an open cleft and the catalytic residues are two glutamate residues [6]. Opposed to family 11, family 10 xylanases have a higher molecular weight, which results in a wider substrate specificity. In addition to the differences in molecular weight, family 11 xylanases have a structure, described as simple single ellipsoidal domain, comprising two β-sheets and a single three-turn α-helix. The catalytic residues are two glutamates [7]. Endo-β-1,4-xylanases act as part of the germination process of cereal kernels and are formed in the aleurone layer. Thus, their endogenous contents in wheat flour are quite low [8]. Xylanases are added to the dough as exogenous enzymes of microbial origin in wheat bread baking. They act on the xylan backbone of arabinoxylans present in the cereal milling products in a random manner [7, 9]. Thus, the degree of polymerization of arabinoxylans is reduced. The products of this enzymatic attack are xylooligosaccharides, xylobiose and xylose [7].
The application of xylanase improves dough handling, dough stability, oven spring, bread volume and staling in wheat bread baking [3, 10].

The impact of a xylanase on bread quality is strongly dependent on its selectivity towards water-extractable or water-unextractable arabinoxylans. Especially xylanase which attacks water-unextractable arabinoxylans and causes a reduction of water-unextractable arabinoxylan content in wheat doughs combined with an increase of solubilized arabinoxylans has a positive impact on bread quality [11]. No significant improvement of bread quality results from the application of xylanase acting on water-extractable arabinoxylans. This results in a reduced water holding capacity of the dough and increased stickiness [4].
The substrate selectivity of the xylanase is highly dependent on the origin of the enzyme. It was shown that bacterial xylanases are more selective towards water-unextractable arabinoxylan and fungal xylanses are more selective towards water-extractable arabinoxylans [11, 12]. The quality improving effect of xylanases in wheat bread making depends on the molecular interaction between arabinoxylans and gluten. Water-unextractable arabinoxylans, as biopolymers, can act like a physical barrier towards gluten network formation during kneading or dough fermentation. This results in a reduced gluten network in wheat doughs and a lower dough stability. Additionally, the water-unextractable arabinoxylans bind huge amounts of water, which is not accessible for the gluten network. This leads to a reduced network formation but also to a higher initial firmness of wheat bread after baking [7, 13]. Xylanases can cause a partial breakdown of arabinoxylans and can enhance the formation of a gluten network which leads to an improved gas retention capacity of the doughs during fermentation and oven spring. Additionally, xylanases cause a better gluten hydration. By partial breakdown of arabinoxylans and their water binding capacity, water is transferred from water-unextractable arabinoxylans to gluten proteins [2, 3, 7]. An overdosage of xylanases in wheat bread baking leads to an extensive degradation of arabinoxylans and thus to a reduced water holding capacity of the doughs. The result are sticky doughs and breads with a poor crumb structure [4].

Another important group of enzymes applied in wheat bread making is the group of lipases. Lipases are enzymes of the group EC 3.1.1.3, so-called triacylglycerol lipases, which act mainly on the ester bonds of triacylglycerols by releasing a fatty acid and a diacylglycerol. Additionally, they are also capable of degrading di- and monoacylglycerols. Since there are several hundreds of lipase amino acid sequences known, there are only few structural similarities. In general, lipases are characterized by a common α/β-hydrolase fold and a conserved catalytic triade with serine as nucleophile, aspartate/glutamate as acidic residue and histidine as basic residue [14]. Lipases are endogenously present in the bran and germ fractions of the wheat kernel. Thus, their contents in whole grain flour are higher than in white wheat flour with low type number and high milling degree [15]. The quality improving effect of exogenous lipase addition in wheat bread making is still under debate. Currently, two different

modes of action are discussed. The first reaction model focuses on the role of polar lipids in gluten aggregation. Several types of lipids are released by lipase during dough mixing and fermentation of dough [16]. Mainly polar lipids are incorporated in the gluten network during mixing [17]. Due to their amphiphilic nature, they can help connect gluten and starch components, as well as promote gluten aggregation itself, while decreasing electrostatic repulsion within the gluten polymer [18, 19]. This leads to a strengthening of the gluten network and to an indirect improvement of gas retention capacity of doughs due to lipases and their reaction products [20, 21]. The volume increasing effect resulting from this mode of action is not observed uniformly [16, 21]. It is rather the second reaction model which describes a direct effect of lipids on gas cell stabilization in wheat breads, as being responsible for the increase in bread volume caused by lipases [22]. According to this model, lipids form a monolayer at the gas/liquid interface of the gas bubbles in the dough, which stabilizes the foam structure in dough and prevents a coalescence of gas bubbles during fermentation. Monogalactosylmonoacylglycerols, N-acyl lysophosphatidylethanolamines and free fatty acids were shown to be the most effective lipase hydrolysis products to affect the loaf volume by stabilizing gas bubbles [21, 23]. Additionally, the conformation of the lipids has a major impact on the stabilizing effect. Hexagonal and cubic arrangements of the lipids in the layer showed a stable condensed monolayer. Both, the type of the hydrolysis product and the conformation of the lipids can be affected by exogenous lipases [21, 22]. Thus, the use of lipases leads to an increase in volume stabilizing the structure of dough and to a uniform and fine crumb structure of the breads [16].

From the enzyme group of oxidoreductases, glucose oxidase (EC 1.1.3.4) is frequently applied in wheat bread making. It is a homodimeric flavoprotein consisting of two identical dimer subunits, which are covalently linked via disulfide bonds. The molecular mass of the protein ranges between 130 kDa and 175 kDA [24]. Glucose oxidase catalyzes the oxidation of β-D-glucose to D-gluconic acid and hydrogen peroxide in the presence of molecular oxygen [25]. Besides glucose, the sugars mannose, xylose and galactose can be oxidized by glucose oxidases, though the reaction rate is lower [26]. Hexose oxidase, another enzyme from the group of oxidases applied in baking, shows a broader substrate specificity. It can catalyze the oxidation of various mono- and oligosaccharides to the corresponding lactones and hydrogen peroxide [25]. Because oxygen is the limiting factor in the reaction of the oxygen-consuming enzymes glucose and hexose oxidase, their activity is restricted to the mixing process and the early stages of fermentation [27]. It is suggested, that the enzymatically formed hydrogen peroxide oxidizes the thiol groups of two cysteine residues to form disulfide bonds [25]. Thus, glucose oxidase alters the structure and the functional properties of the gluten network in wheat dough. The viscoelastic properties of wheat doughs are significantly affected by decreasing

the sulfhydryl content and by creating protein crosslinks through the formation of disulfide bonds [28, 29]. Next to the formation of disulfide bonds in gluten, it was shown that glucose oxidase can affect the formation of ferulic acid bridges in the arabinoxylans and in a limited way the formation of dityrosine crosslinks [26, 29]. This series of crosslinking reactions leads to an increase in dough tenacity and elasticity and a decrease in extensibility [25, 28]. The effects of the modified gluten network are initially noticeable in improved dough handling properties. The bread quality parameters improved by the use of oxidases are mainly the crumb structure and crumb texture as well as the loaf volume [25, 30].

1.1.2 Production of exogenous enzymes applied in wheat bread making

Enzymes used in food production are derived from plants and animal sources or they can be obtained by fermentation using well characterized culturable microorganisms. Considering the entirety of food enzymes on the EU market, about 3% of the food enzymes are isolated from plants, 6% from animal sources and 91% from microbial sources like fungi or bacteria [31]. Wild type strains and genetically modified microorganisms (GMOs) are used for the production of microbial enzymes [32].

Enzymes of microbial origin are produced in an industrial scale via solid-state fermentation or submerged fermentation. During solid-state fermentation microorganisms are grown on solid particles in the absence of a free liquid phase. The liquid phase is retained by the solid particles and thus serves not only as carrier material but also as nutrient source and moisture reservoir [33]. Downstream processing for solid-state fermentation processes is less complex compared to the submerged fermentation process due to the absence of a cultivation broth which must be removed [34]. Solid-state fermentation is often used for the production of fungal enzymes, where stirring in the reactor is not feasible, due to the complex mycelium of the production strain [32]. Submerged fermentation is a form of liquid cultivation, where fermentation takes place with an excess of free water and high oxygen concentrations. Parameters like temperature, pH, aeration and nutritional composition of the medium are constantly controlled. Fermentation is performed on different scales and in different culture methods like batch, fed-batch, perfusion and continuous culture methods [32, 35]. In case of extracellular produced enzymes, downstream processing of the fermentation broth includes several steps of separation, flocculation, filtration, concentration and crystallization. When enzymes are produced intracellular by the corresponding microorganisms prior to the cleanup procedure, a mechanical or enzymatic disruption of the cell walls and membranes is needed [32]. The purified enzyme concentrates, obtained after downstream processing, are subsequently used for the production of commercially available enzyme preparations [35].

It is assumed that about one third of the microbially produced enzymes are derived from GMOs [31]. The advantages of using GMO for the production of enzymes are an increased enzyme production of the microorganism and the avoidance of the production of unwanted secondary metabolites [32].

The microbial production of food enzymes is modified either by genetic modification of the wild type expression strain or by the use of recombinant enzymes [32]. The most frequently used host strains for GMO are the bacterial species *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus amyloliquefaciens* as well as the filamentous fungi *Aspergillus niger* and *Aspergillus oryzae*. In addition to these organisms *Escherichia coli*, *Pelomonas fluorescens* and *Pichia pastoris* are commonly used organisms [35, 36]. For the design of a recombinant production strain, genes encoding the recombinant enzyme are integrated into the host strain via expression vectors. Shuttle plasmids with regulatory elements and selection markers are used as expression vectors. The vector contains at least an origin of replication for independent replication of the DNA within the cell, a multiple cloning site for the integration of the gene of interest and a selection marker [32, 37]. Additionally, promoter and terminator regions are present in the vector as regulatory sequences which control the transcription of the enzyme encoding gene [35]. By the structure of the promoter, the gene expression rate can be increased and thus the enzyme yield [32]. The technique for the insertion of the expression vector into the host strain depends on several properties of the host and the vector. For bacterial hosts, the transformation using cell to cell contact, so-called conjugation, is widely used. Additional to this technique electroporation, component cells and protoplasts are used for the insertion of a vector into a bacterial host. For yeasts and fungi, vector DNA is transformed using incubation with protoplasts [35].

Lately, new technologies for performing genetic modifications have emerged. It was shown that ‘Clustered Regularly Interspaced Short Palindromic Repeat’ (CRISPR) and ‘CRISPR-associated protein-9’ (Cas9) have potential for microbial engineering applications in industrial biotechnology [38]. With this gene engineering technology, the Cas9 endonuclease introduces double-stranded breaks into the target DNA. Via endogenous repair mechanisms the genomic DNA can either be altered by introducing mutations or a specific donor sequence [32]. Most bacteria do not possess the endogenous repair mechanisms and are unable to repair the breaks mediated by CRISPR/Cas9. Thus, wild type strains are killed as a consequence of Cas9 cleavage. CRISPR/Cas9 is a selection tool against wild type cells, and improves the efficiency of bacterial strains after recombineering [39]. For yeasts it was shown, that application of CRISPR/Cas9 can increase the rates of recombination by introducing multiple recombination events within a single transformation step and this leads to an increase in productiveness of the cells [38]. For bacteria, yeasts and filamentous fungi it is possible to

perform gene knockouts via CRISPR/Cas9. Consequently, genes contributing to unwanted reactions during the industrial production of proteins, like foam or secondary metabolites can be disrupted selectively [32, 38]. Filamentous fungi are challenging conventional methods of gene modification like recombination. CRISPR/Cas9 can help to make this process more efficient by the introduction of heterologous sequences in wild type strains [38, 40].

Both techniques, recombination and CRISPR/Cas9 modify the microbial production strain of the enzyme. In contrast to this, methods like rational design, directed evolution and semi-rational design modify the enzyme itself [32]. Rational design requires knowledge about the structure of the target enzyme and its function. By replacement, insertion and deletion of specific DNA regions encoding for amino acids related to properties of the enzyme, the selected characteristics are affected. Directed evolution requires no prior knowledge of the enzyme. It mimics natural evolution in an accelerated in vitro process. Genetic diversity is created by forced mutagenesis, followed by screening for an enzyme with the characteristic properties and its isolation. Semi-rational design is a combination of rational design and directed evolution. Thus, amino acids are mutated in a specific region in the first step. In the second step, the screening and isolation of a suitable enzyme is performed [32, 41]. Temperature optima, thermal stability and pH-tolerance of several enzymes applied in food production were altered with the described enzyme modification methods [31, 41].

1.1.3 Legal principles for the use of enzymes in food

Since 2008 the use of enzymes in food is largely harmonized in the EU by the Regulation (EC) No 1332/2008 on food enzymes and the Regulation (EC) No 1333/2008 on food additives [42]. These regulations are part of the so-called Food Improvement Agents Package and directly applicable in all EU member states [43]. Regulation (EC) No 1332/2008 provides for a list of food enzymes approved for use in food within the EU, the so-called Union list [44]. To add enzymes to the list and thus allow them within the EU market, they must be evaluated. This evaluation process consists of two parts. First, the European Food Safety Authority (EFSA) performs a risk assessment of the enzyme which is published as scientific opinion. In the second part, based on the risk assessment, the European Commission and the member states develop a risk management system [32]. While the Union list is still in preparation, food enzymes currently used in food production must undergo a safety preassessment by the EFSA [42].

The term food enzyme is defined in (EC) No 1332/2008 as a commodity which catalyzes biochemical reactions and is used on a stage in food manufacturing to achieve a technological effect. A technological necessity must be present for its application in food production. Enzymes can be used in food production as processing aids [44]. This term is defined in the

Regulation (EC) No 1333/2008 as a substance not consumed as food, being added during food production for technological reasons and being present as unavoidable technical residues with no technological impact on the final product [45].

Regulation (EC) No 1829/2003 is the basis for the approval of GMOs in relation to food production. Food enzymes, as proteins produced by GMO and used as processing aids, do not fall under this regulation. Thus, the use of a food enzyme obtained from a GMO must not cause the presence of any material from the GMO in the final food product. In contrast, food produced with GMO or consisting of GMO is part of the Regulation (EC) No 1829/2003 and has to go through an approval process and fulfill strict declaration rules [32, 46].

The Regulation (EU) No 1169/2011, the main legal text related to food labeling in the EU, specifies in its Article 20, that food enzymes are excluded from the list of ingredients if they are present due to carry-over effects from other ingredients or if they are used as a processing aid [47]. Enzymes can only be processing aids if they no longer have a technological impact in the final product. This might be the case when enzymes are irreversibly denatured or degraded in the manufacturing process. In this case the denatured or degraded enzymes are unavoidable residues without a technological impact on the final product [48]. It is generally known that heat can inactivate enzymes in an irreversible way. Thus, denatured enzymes present in food which was exposed to a heating step during production, such as bread, are classified as processing aids. Enzymes as processing aids shall not be activated again by changing parameters like pH, temperature or substrate and water availability in the food where they are present [48]. Nevertheless, it is possible, that enzymes are present in foodstuff after processing in a reversible inactivated state. This would be possible if an enzyme is not irreversibly inactivated during baking due to its temperature stability. In this case the Guidance Document on criteria for categorization of food enzymes of the European Commission refers on the impact of the enzyme in the food. A declaration would be necessary if the enzyme is still able to have a technological impact in the food after processing [49].

1.2 Amylases in wheat bread manufacturing

1.2.1 Overview of amylases

Amylases are grouped according to the International Union of Biochemistry and Molecular Biology (IUBMB) nomenclature of enzymes in the group of glucosidases (EC 3.2.1), enzymes hydrolyzing O-glycosyl compounds [50]. The IUBMB categorization is based on the biochemical reaction which is catalyzed. The type of enzyme is coded with a four-component identifier, the EC number, which classifies the enzyme according to a class and several subclasses [51].

In addition to the IUBMB nomenclature of enzymes, glycosyl hydrolases are grouped according to the Carbohydrate Active Enzymes (CAZy) nomenclature. This system groups enzymes which assemble or deconstruct glucans. The grouping is based on structural similarities in the amino acid composition of the enzymes [5, 52]. Most amylases are members of the glycosylhydrolases 13 family. The members of this family share three common domains, whereas the catalytic domain is in the form of a $(\beta/\alpha)_8$-barrel with the active site at the C-terminal end of the barrel strands. The catalytic triade of glycosylhydrolases 13 family enzymes is aspartic acid - glutamic acid - aspartic acid [50].

Several amylases are present endogenously in the wheat kernel, such as α-amylase, β-amylase, pullulanase and α-glucosidase [53]. α-Amylase (EC 3.2.1.1), β-amylase (EC 3.2.1.2) and glucoamylase (EC 3.2.1.3) cleave α-1,4-glycosidic linkages in the starch molecule. α-Amylase as an endoamylase acts randomly on the starch molecule and thus causes an increased release of glucose, maltose and oligosaccharides of varying length. Additionally, this action results in changes of the starch structure [2]. In contrast to α-amylase, β-amylase and glucoamylase are exoamylases which hydrolyze starch molecules starting at the non-reducing end. The result of β-amylase action are β-maltose and β-limit dextrins; glucoamylase converts starch successively into β-glucose [4]. The debranching enzyme pullulanase (EC 3.2.1.42) cleaves α-1,6-glycosidic linkages and thus removes side chains from amylopectin molecules or from limit dextrins [54].

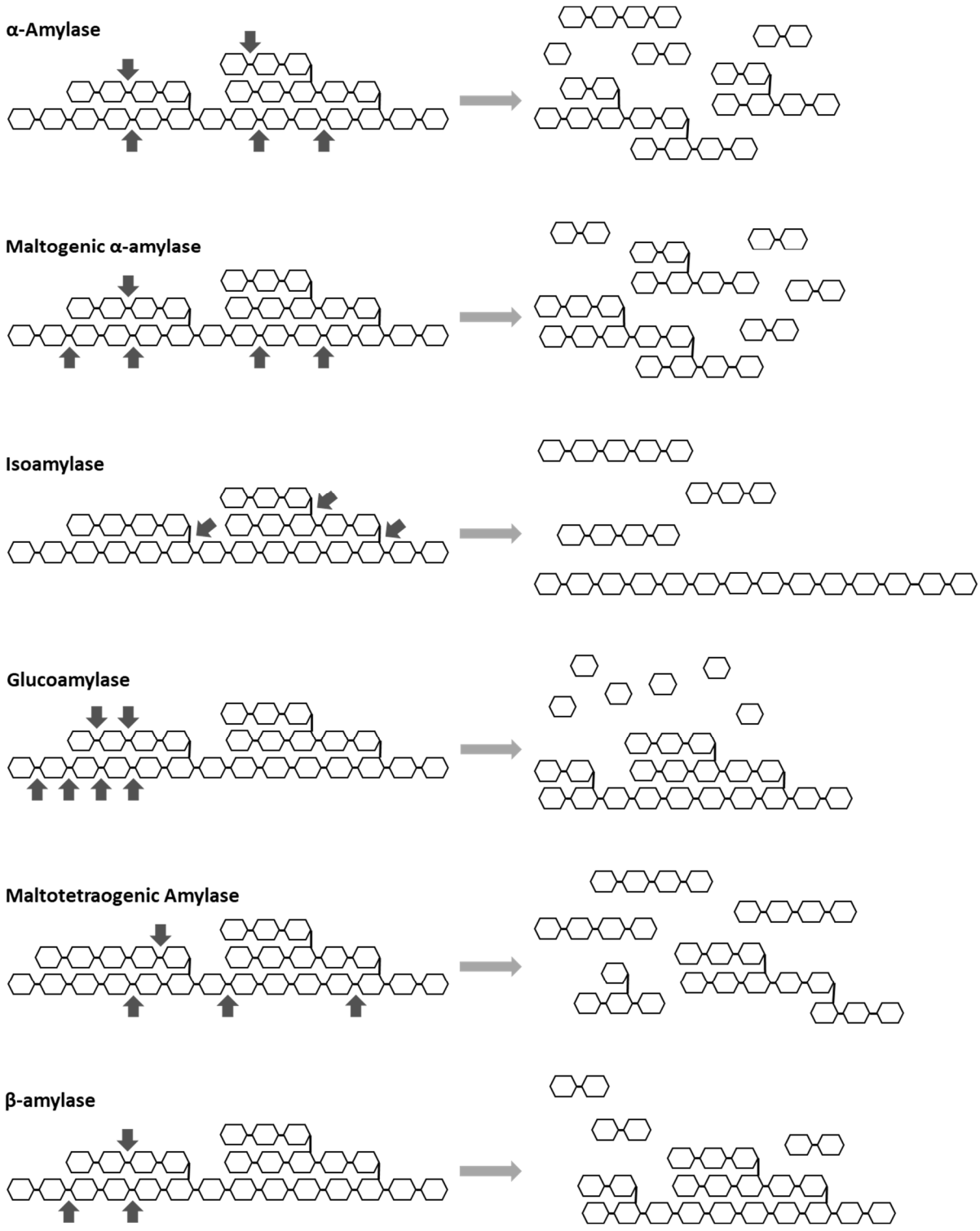

Figure 1: Schematic overview of the amylopectin metabolism of the different amylolytic enzymes. The hexagonal structure represents one glucose unit. Endoaction of α-amylase results in branched and linear dextrins; action of maltogenic α-amylase results to a large extent in maltose; action of isoamylase yields linear dextrins; glucoamylase cleaves off glucose units; action of maltotetraogenic amylase resulting mainly in maltotetraose and β-amylase action resulting in maltose and limit dextrins.

In addition to the endogenous amylases, exogenous amylases from different microbial sources are applied in wheat bread making.

Of the group of exogenous amylases α-amylase is the most frequently used enzyme in bakeries [3]. Other exogenous glucosidases used in bakeries are β-amylase, glucoamylase, maltotetraogenic amylase (EC 3.2.1.60), maltogenic α-amylase (EC 3.2.1.133), and the debranching enzymes pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68) [2, 3]. Except pullulanase and isoamylase, which act on α-1,6-glycosidic linkages, all exogenous amylases cleave α-1,4-glycosidic linkages in the starch macromolecule. Maltotetraogenic amylase removes successive maltotetraose units from the non-reducing end, maltogenic α-amylase removes in the same manner α-maltose units from the starch [55, 56]. Both, maltogenic α-amylase and maltotetraogenic amylase are not purely exo-working enzymes and are shown to perform a limited number of hydrolytic endo-attacks [57]. Different fungi and bacteria are sources of exogenous amylases. Table 1 lists common sources of exogenous amylases applied in baking according to Fraatz et al. [31] and Derde et al. [57].

Table 1: Amylases with corresponding EC-Number and common sources of the enzyme as exogenous amylase in baking

Amylase	**EC Number**	**Production strains/ source**
α-Amylase	3.2.1.1	*Aspergillus niger, Aspergillus oryzae, Bacillus amyloliquefaciens, Bacillus licheniformis, Geobacillus stearothermophilus*
β-Amylase	3.2.1.2	Barley, *Penicillium multicolor*, wheat, soy
Glucoamylase	3.2.1.3	*Aspergillus niger, Aspergillus oryzae*
Pullulanase	3.2.1.41	*Bacillus acido-pullulyticus, Bacillus amyloliquefaciens, Bacillus brevis, Bacillus licheniformis, Bacillus subtilis*
Maltotetraogenic amylase	3.2.1.60	*Bacillus licheniformis, Bacillus subtilis, Pelomonas saccharophila*
Isoamylase	3.2.1.68	*Pseudomonas amyloderamosa*
Maltogenic α-amylase	3.2.1.133	*Bacillus amyloliquefaciens, Geobacillus stearothermophilus*

1.2.2 Endogenous amylases

Endogenous enzymes are present in the ripe cereal grain or are formed during germination and sprouting. This process is related to the uptake of water in the kernel and leads to major metabolic changes including the synthesis of hydrolytic enzymes [58]. The germination process is regulated by plant hormones, of which abscisic acid and gibberellic acid are the most important hormones. Gibberellic acid stimulates growth processes whereas abscisic acid counteracts them. Thus, germination processes are significantly regulated by the concentration and the ratio of the two plant hormones [59]. Both substances are synthesized

in the embryo of the kernel and diffuse to the aleurone where gibberellic acid triggers the aleurone and the scutellum cells to start de novo synthesis and secretion of hydrolytic enzymes like α-amylase and glucoamylase [60, 61]. Hydrolytic enzymes make nutrients available for plant growth and development and thus provide energy to the seedling [61].

α-Amylase has a key role in the process of germination. During the early stages of germination, the enzyme cleaves fragments from the starch and makes them available for other hydrolytic enzymes like glucosidase and β-amylase [53, 62]. Four α-amylase isoforms TaAMY1 – TaAMY4 are known in wheat. They differ in the length of their amino acid chains, their molecular mass (Mr) and their isoelectric point (pI). Table 2 lists the wheat amylase isoforms with their molecular characteristics.

Table 2: Isoforms of α-amylase in wheat with their molecular characteristics. Modified from [63].

α-Amylase isoform	#Amino acids	M_r	pI
TaAMY1	437	47,250	5.98
TaAMY2	439	48,050	5.52
TaAMY3	385	45,470	8.37
TaAMY4	436	47,610	6.01

Although all α-amylase isoforms catalyze the same biochemical reaction, they complement each other during the process of germination by a different point in time of expression. TaAMY1 and TaAMY4 are co-expressed during germination. TaAMY2 is also expressed during germination but independently from TaAMY1 and TaAMY4. The isoform TaAMY3 is expressed during grain development. Further, it is assumed that the substrate specificity and the specificity to inhibitor proteins of the α-amylase isoforms might differ. TaAMY1 and TaAMY4 are suggested to cleave short oligosaccharides more effectively than TaAMY2 and TaAMY3 which are more specific to branched oligosaccharides with more than 20 glucose units [63]. Further, TaAMY1 activity results in an increased release of endogenous proteases during germination [64].

Although glucosidase is capable of directly metabolizing starch, its most effective action can be observed in combination with α-amylase activity. α-Amylase cuts comparatively large fragments off the starch macromolecule which are cut down to glucose units by glucosidase [65]. The glucose generated is converted into sucrose via the hexose phosphate pool and sucrose phosphate synthase, and then is stored in the sieve elements and provides for the main energy supply in the early phases of germination [66].

In contrast to α-amylase and glucosidase, β-amylase is not synthesized de novo during germination. It is present in the ripe cereal kernel in two different forms: A free form, which is

directly extractable with water and a bound form, which is present in the endosperm of the kernel and bound via disulfide linkages to gliadins. During germination the bound form is released by peptidases [67]. Although free β-amylase is present in the ripe kernel, it is not involved in the early stages of germination. The main task of the enzyme is to provide maltose during the growth of the seedling [61, 67].

The contents of the different protein groups, and thus the amylase contents in the ripe wheat kernel are subject to variations due to environmental conditions. Mainly the factors temperature and the amount of rain during the phase of ripening have an impact on amylase content and amylase formation in the kernel [1, 62]. Preharvest sprouting (PHS) and late maturity α-amylase (LMA) are the main reasons for increased amylase levels in wheat. PHS occurs when cereal grain kernels are exposed to rainfall and high levels of humidity after the physiological ripening process in the field. As a result, the synthesis of TaAMY1 and TaAMY2 starts and the cereal grain begins sprouting already on the stalk. The consequence of PHS is an increase in α-amylase activity by a factor of 20 - 40 and, as a result, a considerable loss of quality of the flour made of PHS cereals [62]. LMA results from a genetic defect which affects the synthesis of TaAMY1 and TaAMY4 α-amylases. During the regular ripening process, α-amylase is not synthesized before the kernel has reached its physiological maturity [68]. In LMA wheat, synthesis of TaAMY1 and TaAMY4 α-amylase is triggered before the grain has reached maturity [63, 69]. The accumulation of α-amylase resulting from LMA has no effect on the grain appearance, development or morphology [69]. Opposed to PHS, LMA has limited impact on the bread making quality of wheat flour. Although LMA results in low falling numbers (< 150s), which are an indicator for a low baking quality, no effect of LMA on the quality-related parameters oven spring, loaf volume or crumb firmness could be found [70, 71].

1.2.3 Exogenous amylases

Exogenous amylases have a long history in bread making. The application of malt flours in baking and thus of exogenous amylases is already documented for the late 18th century in central Europe [72]. Nowadays, exogenous amylases are applied in wheat bread making to achieve a targeted technological impact. An overview of commonly used amylases and their sources is shown in Table 1.

α-Amylase has multiple quality-related functions in baking technology. The enzyme has an impact on the fermentation process, on chemical reactions during baking leading to color and aroma formation, and on the staling properties of wheat bread.

The influence of α-amylase on the fermentation process results mainly from the release and the degradation of fragments of the cereal starch. Since the amounts of fermentable free sugars in wheat dough are relatively low, α-amylase provides the majority of fermentable

sugars or their precursors in the dough matrix [73]. By randomly cleaving inner α-(1-4)-glycosidic bonds in the amylose and the amylopectin of wheat starch, glucose, maltose and maltooligosaccharides are released [4]. Maltose and maltooligosaccharides are fermented after the yeast cells shift from glucose to maltose metabolism and thus do not contribute to a fast CO_2 production and a fast increase in loaf volume. However, this action of α-amylases prolongs the productive fermentation time while providing additional substrates such as maltose and maltotriose to the yeast cells [74]. A fast increase of glucose levels in dough, and thus an acceleration of the fermentation rate is achieved by a combination of α-amylase and glucoamylase [75]. In this scenario, α-amylase provides starch fragments which are cut down subsequently to glucose units by glucoamylase. An increasing glucose level in the dough causes a delayed shift in the yeast metabolism from glucose to maltose and as a consequence an increased fermentation rate with a higher loaf volume [74, 75]. The accessibility of starch to amylases is directly related to its condition. The main substrate for amylases in dough is damaged starch, i.e. starch in which the native granular structure is altered. Its amount is dependent on the wheat variety and especially on the milling conditions [10]. The amount of damaged starch and the α-amylase activity are the limiting factors in the release of fermentable sugars from starch. In this case α-amylase has a key role in providing substrates to other hydrolytic enzymes [76].

Sugars formed by the hydrolytic activity of amylases can be precursors in various taste and aroma stimuli forming reactions like the Maillard reaction, the Strecker degradation or the caramelization process. These reactions, which are mainly responsible for the flavor of wheat bread, take place while heat acts on the goods during the baking process in the presence of various reducing sugars [77].

The Maillard reaction starts with a condensation reaction between a reducing sugar and an amino acid, peptide, or other compound with a free amino group. When the reducing sugar is an aldose the product of the condensation is a N-substituted glycosylamine, which is further converted into the 1-amino-1-deoxy-2-ketose, the so-called Amadori product. In case of a ketose being the reducing sugar involved in the Maillard reaction, the product of the condensation is a ketosylamine which is converted during Heyns rearrangement to the Heyns product, a 2-amino-2-deoxyaldose. Neither the Amadori product nor the Heyns product contributes to bread flavor, but they are important precursors which are transformed via different pathways depending on the conditions into flavor-active compounds [78, 79]. Figure 2 provides an overview of the ways for the formation of flavor compounds during the Maillard reaction in case of an aldose sugar as reaction co-product.

Dehydration of the Amadori product, respectively the Heyns product, leads to the formation of furfural, 5-hydroxymethylfurfural, 5-methylfurfural or reductones and dihydroreductones that

give several furan derivatives [79]. Furfural and 5-hydroxymethylfurfural are highly reactive compounds and take part in the formation of melanoidins and other brown colored polymers which are responsible for the browning of the bread crust via condensation and polymerization reactions [78]. Additionally, dihydroreductones can be transformed into aroma-active compounds via Strecker degradation. The Strecker degradation is the oxidative deamination and decarboxylation of an α-amino acid in the presence of a carbonyl compound. In the following, dihydroreductones can be transformed into aldehydes, which take part in the aroma-complex of bread [79].

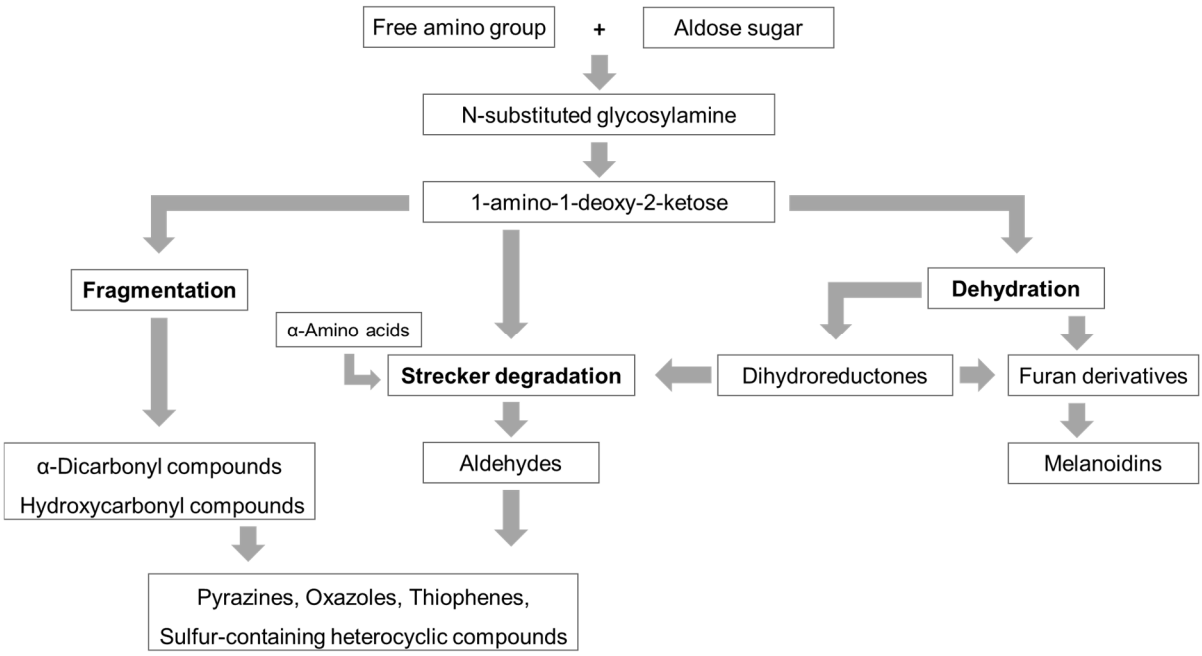

Figure 2: Principal ways of the formation of flavor compounds during the Maillard reaction with an aldose sugar. Figure modified from [79].

Another pathway of the Maillard reaction is the fragmentation of the Amadori- or the Heyns product. This fragmentation leads to a series of α-dicarbonyl and hydroxycarbonyl compounds.

The reaction products of the Maillard reaction and the Strecker degradation interact with each other and lead to the formation of several classes of bread aroma compounds such as pyrazines, oxazoles, thiophenes and sulfur-containing heterocyclic compounds [77, 79].

Since sugars are the most important precursors for dicarbonyls, their content in the dough is important for Maillard reaction products formed during baking [79].

Aroma-active substances resulting from the Maillard reaction are mainly identified in the bread crust, due to heat exposure, but they are also present in the bread crumb. In wheat bread crust, key odorants are 2-acetyl-1-pyrroline (roasted), (E)-2-nonenal (green, tallow), 3-methylbutanal (malty), 2,3-butanedione (buttery, caramel), methional (potato like), and (Z)-2-

nonenal (green). The most important aroma-active compounds in the wheat bread crumb are (E)-2-nonenal (green, tallow), (E,Z)-2,6-nonadienal (cucumber-like), (E,E)-2,4-decadienal (fatty, waxy), 2,3-butanedione (buttery, caramel), methional (potato like), 1-octen-3-ol (mushroom), and (E,E)-2,4-nonadienal (fatty, waxy) [77, 80]. Niehues Birch et al. [80], and Cho and Peterson [77] give a detailed overview of the substances related to bread flavor. The relative amount of substances resulting from the Maillard reaction in bread is described as relatively small, but due to their low odor thresholds they have a huge impact on the overall aroma profile of wheat bread [80]. The amount of Maillard reaction products formed during baking is highly influenced by the baking temperature in the oven, whereby an increasing temperature accelerates the Maillard reaction itself [81].

The second temperature-induced reaction leading to the formation of aroma-active compounds is the caramelization process of sugars. This reaction takes place when sugars on the bread surface are heated above their melting point at about 150°C. From this point on, they react and form colored high molecular weight compounds with a bitter and acidic taste, as well as different carbonyl compounds such as aldehydes and ketones, which also contribute to various odors [77, 81]. Compared to the impact of the Maillard reaction on bread aroma, the caramelization of sugars plays a minor role [79].

Based on their thermal stability, amylases from different sources can provide sugars on different stages of bread making. During the initial stages of baking, sugars are metabolized by yeast and contribute simultaneously to the Maillard reaction. Amylases which have a higher temperature stability than yeast cells, like bacterial amylases, can provide sugars to the dough during later stages of baking. Thus, these sugars are an exclusive substrate for the Maillard reaction and are not metabolized by yeast [82].

Further, the action of amylases on the amylose and amylopectin molecules has an impact on the staling properties of wheat bread. It has been shown that different amylases have different efficiency as antistaling enzymes [83–85]. Maltogenic α-amylase and maltotetraogenic amylase are commonly used as antistaling amylases, but α-amylase also has an effect on the staling and firming properties of wheat bread.

Wheat bread as an unstable multiphase matrix is subject to consistency changing processes during storage: The crispiness of fresh bread crust decreases, whereas crumb firmness and crumbliness increase [86]. These changes, called staling, are caused by water evaporation, immobilization and redistribution processes as well as changes in the ordering structure of the starch polymer. The retrogradation of amylopectin during storage immobilizes freezable water, thus the water content of the amorphous regions in the bread crumb decreases. The immobilized water can no longer plasticize the starch network. Therefore, immobilization of

water contributes directly to crumb firming, although the water content of the bread crumb remains constant during this process [87]. Redistribution of water occurs via water migration from gluten to starch and from crumb to crust during storage. These migration processes reduce the moisture content of the starch and the gluten network, which are responsible for the elasticity of bread crumb. When the moisture content drops below a critical moisture value these biopolymer networks get stiffer and decisively contribute to the firming of bread [87, 88].

Complementary to the processes related to water immobilisation and redistribution, wheat starch rearrangements play the leading role in crumb firming. Amylose crystallizes already during cooling of bread and forms a network which is mainly responsible for the initial firmness of bread crumb [87]. Amylopectin side chains recrystallize over a time span of several days in a process called retrogradation. As a key part of this process, the side chains are packed into tightly packed double-helical structures, which form intra- and intermolecular crystalline regions. Thus, the amorphous gelatinized starch network present in fresh bread crumb is first transformed into a partly crystalline state and subsequently is extended over time into a continuous rigid crystalline starch network [86, 87, 89, 90].

Błaszczak et al., showed that the supplementation of fungal α-amylase in wheat bread baking not only had an effect on the loaf volume, but also on crumb properties such as hardness and elasticity. During storage for five days hardness and gumminess of crumb of wheat breads made with fungal α-amylase were less pronounced than in the control bread without amylase addition. At the same time, elasticity and cohesiveness of the crumb were significantly higher than for the control breads [83]. Since bacterial and fungal α-amylase are mainly endo-acting enzymes, they act on internal α-1,4-glycosidic bonds of the starch polymer. The result is a decrease of the level of long amylopectin starch side chains, which reduces the number of connections between crystallites in the starch network und thus hinders starch recrystallization [84, 91]. The supplementation of wheat doughs with bacterial α-amylase leads to a decrease of the double helical ordering of the starch matrix [85]. This effect does not result from the direct modification of the starch chains via α-amylase, but is traced back to the oligosaccharides resulting from the action of exogenous α-amylase on the starch polymer [92]. It has been reported that oligosaccharides reduce starch recrystallization and might hinder the formation of double helices as well as reduce the mobility of the amylopectin side chains, while acting as antiplasticizers [84, 92]. Compared to the action of exogenous α-amylase on the connections between crystallites in the starch network, the effect of oligosaccharides is described as low [84]. It can therefore be assumed that the freshness enhancing effect of amylases is due to their impact on the starch network in wheat bread crumb. The application of exogenous α-amylases can lead to a fast degradation and thus to a collapse of the starch network resulting in a sticky bread crumb. Via the supplementation of exoacting amylases such

as maltogenic α-amylases this effect can be prevented. Further, maltogenic α-amylases and maltotetraogenic amylases are described as the more efficient antistaling amylases [91, 93].

Maltotetraogenic amylase from *Pelomonas saccharophila* hydrolyzes α-(1,4)-D-glycosidic bonds which leads to the successive removal of maltotetraose residues from the starch polymer. It was shown that the enzyme is not purely an exo-enzyme, but it also shows endo-action and thus a more accelerated starch breakdown than exclusively exo-working amylases [57]. The temperature optimum of the maltotetraogenic amylase from *Pelomonas saccharophila* is at about 60°C and it still showed activity at 90°C. Thus, it can metabolize starch over a long period of time during the baking process [55]. The enzyme mainly acts on the side chains of amylopectin and thus reduces their rearrangement and crystallization. Via the prevention of starch crystallization, bread firming can be delayed [94].

Maltogenic α-amylase from *Geobacillus stearothermophilus* successively cleaves α-maltose units from the non-reducing ends of amylose and amylopectin. The enzyme has its temperature optimum at 60°C, which is comparable to *Pelomonas saccharophila* maltotetraogenic amylase and it has a wide temperature activity range, so that the enzyme can be still active at 90°C [57]. Consequently, the enzyme acts on the starch mainly during the later baking phase and during the cooling phase of the bread [95]. It enhances amylose mobility and network formation, while reducing the molecular weight of amylose [89]. This results in a greater initial firmness of breads manufactured with maltogenic α-amylase compared to breads made with α-amylase supplementation or without any exogenous amylase [95]. Maltogenic α-amylase from *Geobacillus stearothermophilus* is described as amylase with a high degree of multiple attacks. Thus they stay attached rather long to the same starch chain, which results in a slow reduction of the overall molecular weight of the starch [55]. This mode of action results in a systematic shortening of the side chains of the amylopectin molecules, until they become too short to crystallize. Hence, the formation of a permanent, continuous amylopectin network during storage is inhibited [84, 93]. Despite the initial increase in crumb firmness caused by maltogenic α-amylase, its effect on amylopectin leads to a substantial delay in crumb firming [93, 96].

An additional antistaling effect of both maltotetraogenic and maltogenic α-amylases may result from their ability to increase dextrin levels in wheat bread comparable to the action described for the α-amylases above. However, this effect is of minor importance in terms of the antistaling properties of both enzymes [84].

1.2.4 Inactivation of amylases during baking and possible residual activity

The temperature profile of the baking process has a major impact on the kinetics of activation and inactivation of endogenous and exogenous enzymes present in the pastry. Besides the

temperature and its gradient, the parameters moisture content, pH value, the presence of ions and carbohydrates in the bread affect the activity and thermal stability of enzymes [97]. Especially temperature in combination with the moisture content determines the kinetics of the inactivation, whereby the rate of inactivation increases with increasing water content and temperature [98, 99].

The process of enzyme inactivation is divided into two parts: First, reversible denaturation occurs by an unfolding of the enzymes. Thus, enzymes are transformed to an inactive state. By increasing the temperature, the enzymes are denaturated and degraded irreversibly [98, 100]. Denaturation is the loss of tertiary and secondary protein structure, while degradation includes the changes in covalent bonds, e.g. deamidation and oxidation processes of amino acids. These processes are temperature dependent and accelerated at high temperatures. They play a key role in the inactivation of temperature-stable enzymes during heating processes. Mainly the structure of an enzyme determines its temperature stability. It is suggested, that a negatively charged amino acid residue next to the N-terminus of an α-helix has a stabilizing effect due reinforced electrostatic and hydrogen bonds within the helix. Additionally, the substitution of proline for other amino acid residues might have a stabilizing effect in enzymes [101]. The factors improving thermal stability of an enzyme are discussed controversially, but it is assumed, that there is no universal rule for a general protein structure that improves the thermal stability of enzymes [101, 102]. It is known that some enzymes used in baked goods are not completely denatured or irreversibly inactivated during the baking process.

From the group of amylases, a residual activity after baking is specifically described for *Geobacillus stearothermophilus* maltogenic α-amylase and for *Bacillus subtilis* α-amylase in several studies. Lagrain et al. analyzed the impact of these two enzymes on wheat bread and described changes in the saccharide fraction during storage which are a consequence of a residual amylase activity in the bread crumb [95]. A residual activity of the two enzymes after baking was confirmed by Bosmans et al. [93]. Reichenberger et al. also describe a residual amylase activity after baking for *Geobacillus stearothermophilus* maltogenic α-amylase in wheat bread [103]. After incubating maltotetraogenic amylase from *Pelomonas saccharophilia* in a starch solution at 90°C for 30 min Derde et al. still detected a residual activity of the enzyme. As these results are not transferable to the complex matrix of bread, it is still not clarified if the enzyme would be active in wheat bread crumb after baking [57].

Only a few studies investigate the residual activity of fungal amylases in baking. Zhang et al. found a β-galactosidase from *Aspergillus oryzae,* which is still active after baking [97]. The low number of studies dealing with residual activity of fungal amylases in baking can be explained

by the lower temperature stability of amylases from *Aspergillus* compared to bacterial amylases [3].

1.3 Analysis of amylases, their activity and their hydrolysis products in food

1.3.1 Falling number

A widely established method to determine α-amylase activity in cereal products is the falling number according to Hagberg and Perten. Due to its simple and fast feasibility it is a preferred method in the industry [104]. The falling number method is one of the official international standard methods for assessing grain quality and is listed as AACCI Method No. 56-81.03, ICC Standard No. 107/1 and ISO 3093:2009. To determine the index a flour-water suspension is gelatinized by raising the temperature in the sample vessel under controlled stirring. Following the stirring procedure, the stirring rod is dropped through the suspension. Thus, the liquefaction of the starch gel by the α-amylase is meant to be the factor influencing the sinking time of the rod. The stirring time is set to 60 s and the length of the viscometer is 220 mm. The falling number is defined as the sum of the stirring time and the time it takes the rod to sink through the suspension [105]. The index is proportional to the viscosity and inversely proportional to the α-amylase activity. Falling numbers between 250 s and 450 s stand for normal α-amylase activities and should represent wheat flours with good baking properties [71, 106]. It was shown, that the falling number has a high level of repeatability and shows associations with the α-amylase activity of wheat samples [106, 107].

However, the applicability of the falling number as predictive value for the α-amylase activity or the baking quality of wheat flour is currently under debate. Basically, the method is the measurement of an effect and not the direct determination of α-amylase activity. It neglects factors such as the rheological properties of the starch or variety-specific starch properties [108]. Further, it was shown, that the association between falling number and α-amylase activity is indeed present, but not strong enough to consider the falling number a highly robust predictor of α-amylase activity [107]. In addition to the properties of starch, the grain protein content and composition as well as the genotype influence the falling number [104]. For the prediction of LMA or PHS, the falling number was shown to be unsuitable. Based on the falling number it was not possible to differentiate between the two genetic defects in endogenous α-amylase levels and regarding PHS the test was not effective in quantitating a level of sprouting [104, 109]. Further, a clear absence of a correlation of falling number values with LMA-related α-amylase activities was described [70].

Nowadays it is assumed, that the isoform of α-amylase present in a wheat sample plays a key role for the baking quality, i.e., a high α-amylase level itself does not result in a poor baking

quality. It was rather shown, that an increase in the α-amylase isoforms TaAMY1 and TaAMY3 results in a positive impact on the bread quality parameters volume and browning [64, 70].

1.3.2 Activity of the different amylases

Although enzymes usually occur in very low concentrations they can be identified, and their activity can be quantified by their catalytic reaction. Such a relatively simple indirect approach is not transferable to other proteins present in biological samples such as nucleic acids or structural proteins. Despite the relatively simple procedure, enzyme assays are only reliable and reproducible if the conditions are strictly standardized. For the activity of enzymes the pH, temperature, ion strength and substrate availability are described as the most important factors [110].

Different enzyme assays have been established for the determination of the activity of amylases. There are several methods that measure the amount of substrate, for example starch, in a sample after amylolytic activity or the amount of reducing sugars; most of them are based on spectroscopic techniques [111]. Nowadays, chromogenic methods to determine amylase activity, are used more frequently. Here, a soluble dye or a chromogen is coupled to a maltooligosaccharide as substrate. Most of them are solution-based fluorometric or colorimetric assay procedures. Assay procedures are established for α-amylase, β-amylase, debranching enzymes and amyloglucosidase working with a 4-nitrophenyl group coupled to a maltooligosaccharide. The structure of the oligosaccharide depends on the substrate affinity of the analyzed amylase [112–115]. Since amylases are only capable of cleaving bonds between adjacent glucose units, these 4-nitrophenol-based assays include an α-glucosidase to cleave off the 4-nitrophenol group from the amylase reaction products, mostly short oligosaccharides. The α-glucosidase involved in the assay procedure must not act on the intact substrate. Thus, these assays include two steps of enzymatic metabolism. [111]. Figure 3 shows the reaction scheme of the Ceralpha α-amylase assay. The release of the 4-nitrophenyl ion results in a yellow-colored solution with a measurable extinction correlating with the respective amylase activity.

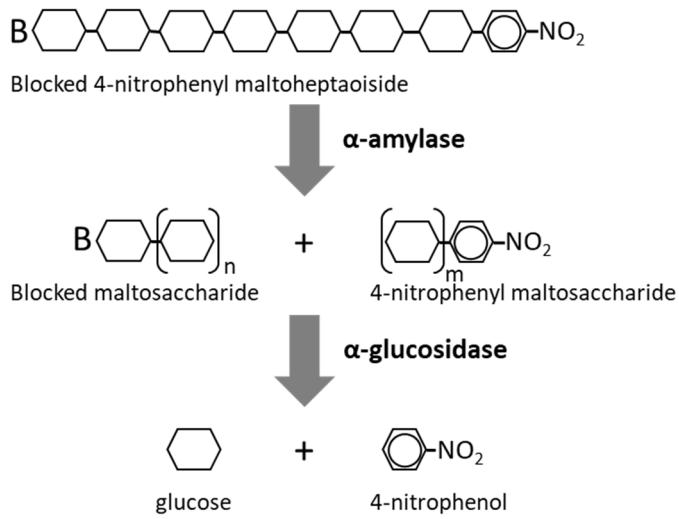

Figure 3: Reaction scheme of the Ceralpha α-amylase assay. α-Amylase cleaves bonds within the blocked 4-nitrophenyl maltoheptaoiside. In the second step the α-glucosidase cleaves 4-nitrophenyl off the non-blocked reaction product.

Chromogenic methods are described as fast and they can be adapted to microtiter plates, which ensures high sample throughput. The methods were shown to have high accuracy and reproducibility [116].

Based on the substrate turnover during the incubation time of the assay, the activity of the amylase can be expressed in units of activity or Katal (kat). One unit is defined as the enzyme amount converting 1 µmole of substrate per minute and it is still very popular although Katal is the measuring unit for enzyme activities according to the SI system. One Katal is defined as the activity of converting 1 mol of substrate per second [110].

1.3.3 Amylase hydrolysis products

Several methods to identify and quantify mono-, di-, oligo- and polysaccharides have been published, whereby different analytical techniques have been used, for example capillary electrophoresis [117], LC-MS [118, 119], gas chromatography (GC)-MS [119, 120], high-performance liquid chromatography (HPLC) coupled with different detection methods [117, 121] and high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) [76, 122]. Due to the high sensitivity and the ability to analyze a large number of different carbohydrates in one run, HPAEC-PAD gained popularity in carbohydrate analysis [122].

Carbohydrates are weak acids and thus their hydroxyl groups are partially transformed into oxyanions at high pH. Sodium acetate solution in combination with sodium hydroxide solution is routinely applied in anion exchange chromatography to realize a high pH value in the mobile phase. The anions are bound to the stationary phase, where the order of retention is negatively correlated with the pK_a value. During detection via PAD the carbohydrates are oxidized on the

surface of a gold electrode. The electrical current generated by their oxidation, results in the signal which is measured during detection [121].

HPAEC-PAD experiments with cereal-based samples require a simple work-up procedure. The extraction of carbohydrates can be performed either with water or water-alcohol solutions with varying concentrations [122–124]. High alcohol concentrations were shown to reduce the amount of starch being extracted. Additionally, endogenous enzymes can be inactivated by alcohols in the extraction solution to avoid further hydrolysis of the extracted sugars. A simple clean-up procedure of the extract including filtration and evaporation of the alcohols is sufficient for the HPAEC experiment [122].

1.3.4 Discovery-driven proteomics

The field of proteomics deals with the large-scale determination of genetic and cellular functions at protein level using a whole range of analytical techniques. Mass spectrometry-based methods have become very popular as a tool to analyze complex protein samples. This was made possible by the establishment of gene and genome sequence databases [125]. Mainly liquid chromatography (LC) systems coupled to a mass spectrometer (MS) are used in MS-based proteomics. The MS system generally consists of an ion source, a mass analyzer and a detector. Popular ionization techniques are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI); mass analyzers applied in proteomics are time-of-flight (TOF) or triple quadrupole (QQQ) analyzers. MALDI-TOF-MS is routinely used for the characterization of intact proteins, whereas discovery-driven and targeted proteomics, focusing on tryptic peptides, are performed with LC-ESI-TOF-MS and LC-ESI-QQQ-MS systems, respectively [125, 126].

In general, the discovery driven-proteomics approach is chosen when a mixture of different proteins should be analyzed. Therefore, proteins are extracted or isolated followed by a hydrolysis step, during which a sequence specific protease like trypsin cleaves the proteins into specific peptides [127]. The peptide mixture is separated via LC to reduce its complexity and transferred to the MS.

Typical discovery-driven proteomics experiments are designed as data-dependent acquisition (DDA) experiments. Therefore, within a TOF-MS scan, precursor ions are detected and selected according to DDA criteria like intensity in the first quadrupole. These precursor ions are fragmented in the collision cell of the system. In the following step the resulting product ions are detected based on their mass-to-charge ratio by a TOF-analyzer [128]. Identification of the detected peptides is performed *in silico*. The spectra of the detected peptides are matched with databases containing proteins and their simulated peptides. These database searches are performed with protein databases available online [129] which are processed by

search engines implemented in bioinformatics software tools such as MaxQuant [130]. Figure 4 illustrates the workflow of a discovery-driven proteomics experiment in a simplified form.

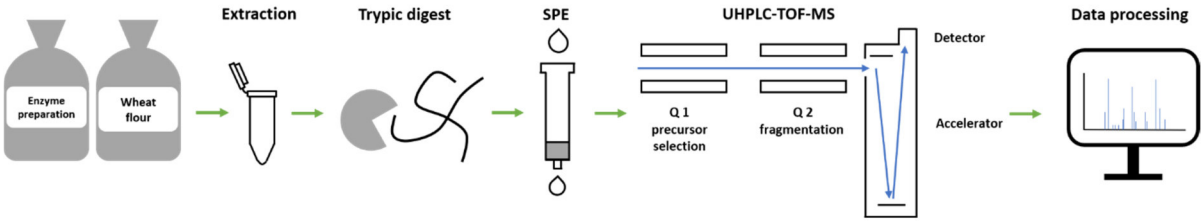

Figure 4: Workflow of the discovery-driven proteomics approach. Sample preparation including the main steps protein extraction, tryptic digest and sample clean-up via Solid Phase Extraction (SPE). Proteomics experiment is performed on a UHPLC-TOF-MS system, data processing includes the identification of peptides and corresponding proteins in the samples with MaxQuant.

In addition to DDA experiments, lately so-called data-independent acquisition (DIA) experiments have become more popular in proteomics. In the DIA mode, all ionized compounds of a sample that fall in a specified mass range are fragmented. Thus, DIA provides a huge amount of information, but comes along with a large increase in the complexity of the data evaluation process compared to DDA experiments [131].

2. Aim of the work

Exogenous enzymes are used routinely in wheat bread making. They are added during dough preparation with the intention of acting as technological processing aids on various stages of bread making. Among these enzymes, amylases are the most important representatives. They are added to improve volume and color of wheat bread as well as decrease bread staling. As processing aids, their activity has to be limited to the production process according to European legislation. Otherwise, if there is a technological function on the final product, they are no longer processing aids and have to be labeled on the product. Until now, it was assumed that amylases in wheat bread making are inactivated during the baking process and that they have no technological function in the final product. Consequently, there is little information available about a residual activity in wheat bread.

The aim of our study was to elucidate the relationship between the protein composition of amylase preparations, changes in sugar composition of the breads during storage and a possible residual activity of the enzyme.

For this purpose, we first identified and quantitated different amylases (α-amylase, maltotetraogenic amylase and maltogenic α-amylase) in common preparations used in baking via label-free discovery-driven mass spectrometry. This step should generate knowledge of the proteins present in the preparations and should enable the assignment of an activity to each amylase.

To study the action of the amylases in the matrix bread, baking experiments with the application of the amylase preparations were performed in the second step. By monitoring mono-, di- and oligosaccharides in these model breads we got insights in the saccharide composition and its development during storage. Further the activity of amylases was determined with suitable colorimetric assays to detect a possible residual activity in the bread crumb. The combination of these two methods allows to establish the relationship between characteristic molecular changes in the bread crumb and an amylolytic residual activity in the breads.

Thus, the combination of these methodological approaches and the resulting novel findings shall provide fundamental knowledge about the application of amylases in baking, their possible residual activity and the resulting changes in the sugar spectrum.

3. Results

3.1 Impact of exogenous α-amylase on sugar formation in straight dough wheat bread

In a set of five different commercially available α-amylase preparations, we identified the amylases responsible for the amylolytic activity of the preparations by a discovery-driven proteomics approach. Further, baking experiments were performed to identify a residual amylase activity in the bread crumb after baking. Within the scope of HPAEC-PAD experiments, mono-, di- and oligosaccharides in the model breads were identified and their development during storage was monitored. Gerold Rebholz developed the concept of the experimental setup, planned, performed the experiments and carried out the data analysis and data interpretation.

In the five α-amylase preparations, a total of three different α-amylases were identified. Two preparations contained bacterial α-amylase from *Bacillus amyloliquefaciens* and *Thermoactinomyces vulgaris*, respectively. In three preparations, amylases from *Aspergillus oryzae* were identified, one α-amylase and one glucoamylase. The preparation containing *Bacillus amyloliquefaciens* α-amylase showed the largest impact on the sugar spectrum of the model breads. During the storage time of up to 96 h the total sugar content increased by 408.6% compared to the control bread without amylase addition. Moreover, this preparation showed a residual activity of 4.01% based on the applied activity. The fungal preparations showed changes in the sugar composition of bread during storage, which were, however, smaller compared to the control bread. For the fungal preparation no residual amylolytic activity was detected in the bread crumb.

Our work provides new insights into the processes of the saccharide fraction of wheat bread supplemented with α-amylase. The combination of this part of the work together with the proteomics-based approach of the enzymatic activity allowed to establish a link between exogenous α-amylase and its activity during wheat bread storage. Thus, our research provided the basis for a further investigation of the technological function in the bread crumb.

Additionally, Gerold Rebholz designed the figures, wrote the manuscript and revised it according to the comments of the co-authors and reviewers.

European Food Research and Technology
https://doi.org/10.1007/s00217-020-03657-y

ORIGINAL PAPER

Impact of exogenous α-amylases on sugar formation in straight dough wheat bread

Gerold Felix Rebholz[1] · Karin Sebald[2] · Sebastian Dirndorfer[2] · Corinna Dawid[2] · Thomas Hofmann[2] · Katharina Anne Scherf[1,3]

Received: 12 October 2020 / Revised: 9 November 2020 / Accepted: 14 November 2020

Abstract

The use of bacterial or fungal α-amylases is common in wheat bread production to improve several quality-related parameters such as loaf volume, crust color or staling behavior. To study the impact of exogenous α-amylases on straight dough wheat bread, we quantitated mono-, di- and oligosaccharides and residual α-amylase activity in bread crumb during storage for up to 96 h. Discovery-driven proteomics of the five α-amylase preparations studied showed that only a few different amylases per preparation were responsible for the hydrolytic effect. Compared to the control, the supplementation with α-amylase from *Bacillus amyloliquefaciens* in wheat dough preparation led to major changes in the sugar composition of bread crumb during storage with the formation of oligosaccharides like maltopentaose, maltohexaose, maltoheptaose, and maltooctaose. A residual activity corresponding to 4.0% of the applied activity was determined in the breads prepared with α-amylase from *B. amyloliquefaciens*, but no residual activity was detected for any of the other fungal or bacterial α-amylases from *Aspergillus oryzae* or *Thermoactinomyces vulgaris*. Whether the detected residual activity is related to the characteristics of bread staling or bread crumb properties must be clarified in further studies.

Keywords Amylase · Bread · Enzymatic activity · Maltose · Oligosaccharides · Wheat

Introduction

Amylases are present endogenously in wheat flour, but their activities vary greatly depending on the type and variety of grain, the environmental conditions during cultivation, and the state of maturity at harvest [1]. It is generally accepted that the amount of amylases present endogenously in wheat flour is too low to have a technological impact during the manufacturing of wheat bread except in cases of pre-harvest sprouting or late-maturity amylase activity. Considerable levels of β-amylase are present in unmalted wheat flour, while its α-amylase content and associated activity are low. α-Amylases (EC 3.2.1.1) are the most widely used enzymes in baking and play an important role due to their multiple quality-related functions in baking technology [2]. The quality-improving effect of the different α-amylases used in baking is mainly based on their influence on the fermentation process, the influence on chemical reactions during baking leading to color and aroma formation and/or antistaling mechanisms [3, 4].

α-Amylases predominantly catalyze endohydrolysis of *α*-(1–4)-D-glycosidic bonds in the amylose and amylopectin molecules of wheat starch, but may also act as α-exoamylase, generating α-maltose from the non-reducing end. Thus, α-amylases cause an increased release of α-configured glucose, maltose, and oligosaccharides of varying length resulting in changes in the starch structure [5]. The addition of α-amylase to wheat doughs improves the fermentation rate by providing additional substrates such as maltose and maltooligosaccharides to the yeast cells. This leads to a prolongation of the productive fermentation due to the fact that

✉ Katharina Anne Scherf
katharina.scherf@kit.edu

1 Leibniz-Institute for Food Systems Biology at the Technical University of Munich, Lise-Meitner-Strasse 34, 85354 Freising, Germany

2 Chair of Food Chemistry and Molecular Sensory Science, TUM School of Life Sciences, Technical University of Munich, Lise-Meitner-Strasse 34, 85354 Freising, Germany

3 Department of Bioactive and Functional Food Chemistry, Institute of Applied Biosciences, Karlsruhe Institute of Technology (KIT), Adenauerring 20 a, 76131 Karlsruhe, Germany

these sugars can be fermented by yeast shifting from glucose to maltose metabolism [6, 7].

In addition, the sugars formed by the hydrolytic activity of amylase can be substrates in various taste and aroma stimuli forming reactions. Especially, the caramelization process, the Maillard reaction, and the Strecker degradation are reactions that take place during the baking process under the influence of heat and in the presence of various reducing sugars. They are mainly responsible for the flavor and taste of wheat bread [8].

The action of α-exo-amylases on the amylose and amylopectin molecules has a major impact on the staling properties of wheat bread. The degradation of the amylopectin side chains by α-amylase hinders starch recrystallization and thus reduces the firming rate of starch [9]. Thermostable and intermediate temperature stability bacterial α-amylase had an antistaling effect due to its ability to decrease the double-helical ordering of the starch matrix and this hindered starch retrogradation [10]. In addition to the antistaling effect resulting from the action on the three-dimensional structure of starch, α-amylase might show an antistaling effect due to the release of oligosaccharides from starch [11]. Oligosaccharides with an average degree of polymerization of 5.1–6.2 for *Bacillus subtilis* α-amylase and with 2.6–2.8 for *Geobacillus stearothermophilus* maltogenic α-amylase might act as antiplasticizers, which reduce the mobility of amylopectin side chains and hinder the formation of double helices. However, despite a high amount of oligosaccharides, recrystallization was hardly reduced in wheat bread crumb [12].

During the baking process, the amylases are first reversibly unfolded and converted into an inactive state. In the second step after extended heat exposure, the proteins are irreversibly denatured [13]. The kinetics of inactivation is strongly correlated to the water content and heating rate in bread [14]. It is known that amylase from *B. subtilis* and *G. stearothermophilus* might be partly active after baking in wheat bread crumb, whereas the thermal stability of fungal amylase is lower than that of cereal amylase [15, 16].

In the European Union the use of enzymes in food production must be in compliance with the Regulation (EC) 1332/2008 [18]. In consideration of the Regulation (EU) No 1169/2011 on the provision of food information to consumers, enzymes used as processing aid are not classified as food ingredients [19]. Thus, they do not have to be labeled. In the case of residual activity of enzymes in food, it must be clarified if the residual activity has a technological function in the final product. If there is a technological function in food, the enzymes are classified as ingredients and have to be labeled [18, 19]

In our study, we focused on the impact of exogenous α-amylases in straight dough wheat bread making. The main goal was to elucidate the relationship between the composition of commercial α-amylase preparations, changes in bread during storage and possible residual activity of the enzyme. Via label-free discovery-driven mass spectrometry, we aimed to identify and quantitate α-amylase in common preparations used in baking. By monitoring mono-, di- and oligosaccharides in the bread we got insights into the saccharide composition and its development during storage. The combination of these two methods with a colorimetric assay for the activity in bread after baking gave information about the cause and effect of molecular changes related to residual amylolytic activity in straight dough wheat bread.

Materials and methods

Chemicals and flours

All chemicals and solvents were at least HPLC-grade or higher. Water was purified using an Arium 611VF water purification system (Sartorius, Goettingen, Germany). Trypsin (from bovine pancreas, TPCK-treated, ≥ 10,000 BAEE U/mg protein), sodium hydroxide solution (50–52%), and anhydrous sodium acetate (≥ 99%) were obtained from Sigma-Aldrich (Steinheim, Germany). α-Amylase assay reagent (4-nitrophenyl α-D-maltoheptaoside (blocked), plus excess α-glucosidase and glucoamylase) and β-amylase assay reagent (4-nitrophenyl β-D-maltotrioside, plus excess thermostable β-glucosidase) were purchased from Megazyme (Bray, Ireland). D-(+)-glucose (≥ 99.5%), D-(−)-fructose (≥ 99%), sucrose (≥ 99.5%), maltose monohydrate (≥ 99%), maltotriose (≥ 90%), and maltoheptaose (≥ 70%) were obtained from Sigma-Aldrich. Maltotetraose (≥ 97%), maltopentaose (≥ 90%), maltohexaose (≥ 95%) and maltooctaose (≥ 80%) were purchased from Santa Cruz (Dallas, USA). Wheat flour type 550 was provided by Rosenmühle (Ergolding, Germany).

Amylase preparations

Five different α-amylase preparations commonly applied in bread making were used. The enzymes originated from *Bacillus amyloliquefaciens* (A), *Thermoactinomyces vulgaris* (B), and *Aspergillus oryzae* (C, D and E). The preparations, their ingredients and dosage specified by the manufacturer are listed in Table 1. The activity was determined by means of the α-amylase assay procedure described below.

Enzyme extraction from amylase preparations and determination of the protein content

The amylase from the preparations was extracted according to Uhr et al. [19]. A sample quantity corresponding to 0.5 g of dry matter was extracted using 5.0 mL of extraction

Table 1 Composition of the enzyme preparations as indicated by the manufacturer, dosage recommended by the manufacturer, and activity of the amylase added to the dough

Preparation	Enzyme	Other ingredients	Dosage [mg/kg based on flour]	Activity [nkat/g_{dm}]
(A)	Bacterial α-amylase	Wheat starch, edible oil	50	1.77
(B)	Bacterial α-amylase	Wheat flour, sunflower oil	600	62.74
(C)	Fungal α-amylase	Wheat flour, wheat starch, dextrin	10	6.52
(D)	Fungal α-amylase	Wheat flour, dextrin	25	77.39
(E)	Fungal α-amylase	Wheat flour	50	27.08

buffer (0.1 mol/L ammonium bicarbonate, 4.0 mol/L urea, and 0.005 mol/L dithiothreitol (DTT) (pH 8.0) in water). The extraction procedure was performed at room temperature by stirring for 30 min. Following the extraction step, the extract was centrifuged (20 min; 20 °C; 3750×*g*) and the supernatant was collected for further analysis.

The proteins were quantitated in the extracts of the preparations by an RP-HPLC method using a Jasco-XLC System (Jasco, Pfungstadt, Germany) with an Acclaim 300 C_{18} column (3 µm particle size, 30 nm pore size, 2.1 mm × 150 mm; Thermo Fisher Scientific, Waltham, USA) equipped with a security guard ultra column of the same type. The column temperature was 60 °C; the injection volume was 10 µL. Elution solvents were (A) 0.1% aqueous trifluoroacetic acid (TFA) and (B) acetonitrile containing 0.1% TFA. A linear gradient was applied as follows: 0.0 min, 0% B; 0.4 min 0% B, 0.5 min, 24% B; 15.0 min, 56% B; 15.1 min, 90% B; 19.1 min, 90% B; 19.2 min, 0.0% B; 35.0 min, 0.0% B. The flow rate was 0.2 mL/min. Detection was carried out by measuring the UV absorbance at 210 nm [21]. Control of the system and the evaluation of the chromatograms were done with the software ChromPass (Jasco). Quantitation of the protein content of the samples was performed by means of external calibration using reference gliadin from the Working Group on Prolamin Analysis and Toxicity (PWG-gliadin) [22]. All determinations were done in triplicate.

Sample preparation for ultra-high-performance liquid chromatography time-of-flight mass spectrometry (UHPLC–TOF–MS)

Amylase from wheat flour and from the preparations was extracted according to Uhr et al. [19]. Following the extraction and centrifugation an appropriate volume of the supernatant was diluted to a protein content of 0.5 mg/mL in 0.3 mL and 0.1 mL Tris–HCl (1.5 mol/L) and 0.1 mL 1-propanol was added. Reduction was performed by adding 0.05 mL tris(2-carboxyethyl)phosphine (0.05 mol/L tris(2-carboxyethyl)phosphine in 0.5 mol/L Tris–HCL, pH 8.5) and incubating for 30 min at 60 °C. The cysteine residues of the proteins were alkylated using 0.1 mL chloroacetamide solution (0.5 mol/L chloroacetamide in 0.5 mol/L Tris–HCl, pH 8.5) per sample for 45 min at 37 °C in the dark. After the alkylation step, the samples were evaporated to dryness using a rotary vacuum concentrator (37 °C, 3 h, 800 Pa). Tryptic hydrolysis was carried out by reconstitution of the samples in 0.5 mL trypsin stock solution with trypsin:substrate ratio of 1:20 (w/w) (0.04 mol/L urea in 0.1 mol/L Tris–HCl, pH 7.8). The samples were incubated at 37 °C for 16 h. Hydrolysis was stopped by heating the samples at 95 °C for 8 min. Following the tryptic digestion, the samples were purified via solid-phase extraction using 100 mg Discovery DSC-18 tubes (Merck, Darmstadt, Germany). The tubes were activated using methanol (1 mL), equilibrated with acetonitrile/water/formic acid (FA) (80:20:0.1; 1 mL) and washed with acetonitrile/water/FA (2:98:0.1; 5 × 1 mL). After washing the tubes, the samples were loaded on the tubes and the cartridges were washed again as described before. Elution of the peptides was performed with acetonitrile/water/FA (40:60:0.1). The eluate was dried using a rotary vacuum concentrator (37 °C, 4 h, 800 Pa), reconstituted in 500 µL FA and filtered (0.45 µm; wwPTFE). The analysis of three independent technical replicates each confirmed that the chosen workflow led to reproducible results in terms of confident qualitative peptide and protein identification.

Discovery-driven proteomics using UHPLC–TOF–MS

UHPLC−TOF−MS measurements were carried out on a Sciex TripleTOF 6600 mass spectrometer (Sciex, Darmstadt, Germany) connected to a Shimadzu Nexera X2 system (Shimadzu, Kyoto, Japan) operating in the positive electrospray ionization (ESI) mode. Control of the instrument and data acquisition were performed using AnalystTF software (Version 1.7.1, Sciex). Chromatography was performed on a 150 × 2.1 mm, 1.6 µm BioZen Peptide PS-C_{18} LC column (Phenomenex, Aschaffenburg, Germany) equipped with a security guard ultra column (2 × 2.1 mm) of the same type. Elution solvents were (A) 1% aqueous FA and (B) acetonitrile containing 1% FA. The gradient was performed at a flow rate of 0.35 mL/min starting at 0 min, 5% B; 5 min, 5% B; 60 min, 40% B; 65 min, 100% B; 69 min, 100% B; 70 min, 5% B; and 75 min, 5% B. The temperature of the column oven was set to 40 °C, the injection volume was 10 µL.

For the experiments, the ion spray voltage was set at 5500 eV, the source temperature was 550 °C, the nebulizing gas was at 55 psi, the heating gas was at 65 psi and nitrogen served as curtain gas at 35 psi.

In the information-dependent acquisition mode (IDA), a TOF−MS survey scan was acquired from *m/z* 400 to 1000 using an accumulation time of 250 ms, collision energy (CE) of 10 V and a declustering potential (DP) of 80 V. Product ion spectra for the 12 most abundant compounds were recorded in the high-resolution mode for 70 ms within the *m/z* range of 100 − 2000 using a DP of 80 V and CE of 40 V, with CE spread of 15 V. IDA criteria for the precursor ion included intensity of > 100 counts/s. Q1 resolution was set to 0.7 Da.

Protein identification using MaxQuant

A search workflow using MaxQuant (version 1.6.10.43) was developed for the identification and relative quantitation of proteins (Fig. 1). The MS raw files were used as input in the MaxQuant software for different searches against databases derived from UniProtKB. The first step of data evaluation was the identification of enzymes from the class of glycosylases (EC 3.2.-.-). A database was built with all proteins belonging to the class EC 3.2.-.- from *Triticum aestivum* and from 14 microorganisms relevant for the production of food-grade amylases according to the European Food Safety Authority and the US Food and Drug Administration [23, 24]. This resulted in a database containing 5550 proteins (Download: 01.07.2019). To identify all possible proteins present in the sample, in the second step a database containing the proteome of the identified organisms was used, respectively. In the final third step, the relative quantitation of the proteins was performed using the intensity based absolute quantitation (iBAQ) algorithm implemented within MaxQuant. Via iBAQ, the iBAQ intensities of different proteins within one sample can directly be compared to each other, and the relative concentrations of proteins can be determined [25]. Therefore, for each sample, an individual database was built containing all the identified proteins from the second step. A total sum normalization of iBAQ protein intensities between samples was performed to correct for different total protein injection amounts. The parameters were set as follows for all MaxQuant searches: Variable modification included oxidation of methionine and N-terminal protein acetylation. Carbamidomethylation on cysteines was specified as fixed modifications. Trypsin was set as a proteolytic enzyme with up to two allowed missed cleavage sites. Match-between-runs was enabled with a matching time window of 0.7 min and an alignment time window of 20 min. Results were filtered for a minimal length of five amino acids and 1% peptide and protein false discovery rate.

Baking experiments

Baking experiments were performed on a 50 g flour scale. The baking recipe was: Wheat flour type 550 (50 g, based on 14% moisture), 2.0 g baking soda, 0.25 g sodium chloride,

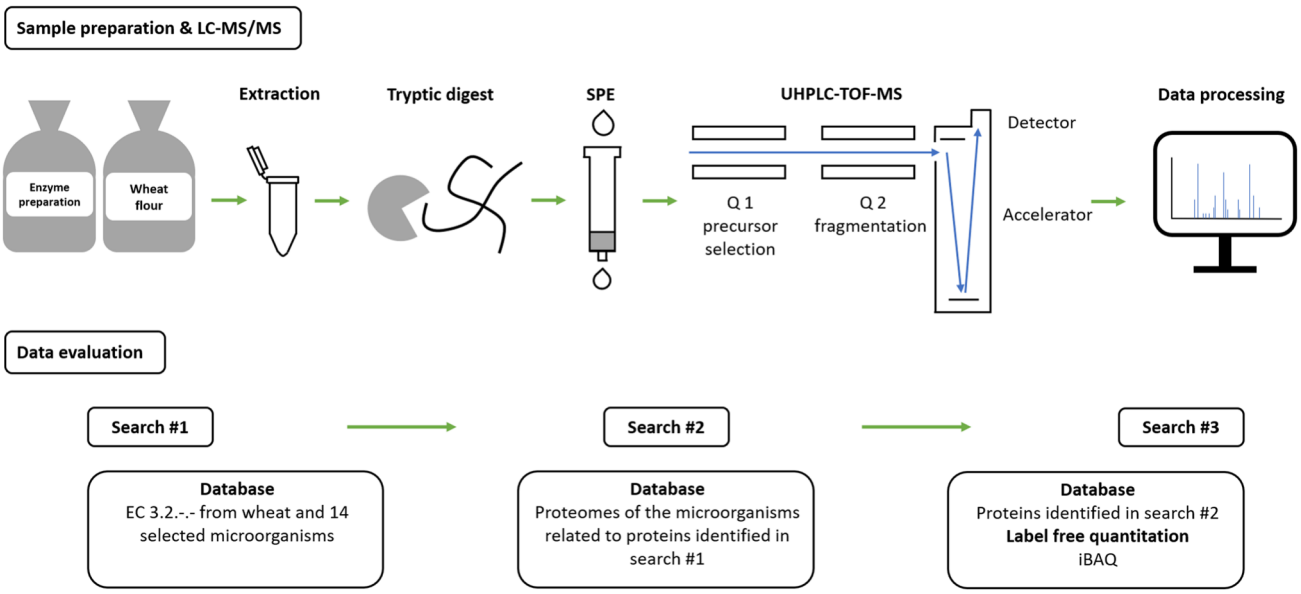

Fig. 1 Workflow illustrating the procedure from sample preparation to UHPLC–TOF–MS analysis and data evaluation using three subsequent search strategies to identify the peptides and the corresponding proteins present in the amylase preparations and the wheat flour. iBAQ, intensity-based absolute quantitation; SPE, solid-phase extraction; UHPLC-TOF–MS, ultra-high-performance liquid chromatography time-of-flight mass spectrometry

bulk liquid as determined in the Farinograph-E [26]. To obtain a pH comparable to yeast-leavened wheat bread (pH 6) water as the bulk liquid was replaced by a buffer solution with 0.1 mol/L citric acid monohydrate and 0.1 mol/L trisodium citrate dihydrate (pH 2.75). Baking soda was added 1 min before the end of the kneading time. The amylase preparation was added to the dough in powder form and dosed as shown in Table 1. The dough was kneaded in the Farinograph-E at 60 rounds per min to optimum consistency [26]. After kneading the dough was rounded for eight cycles and placed in the proofing cabinet, where the first dough rest took place for 20 min in a water-saturated atmosphere at 30 °C. Then, the dough piece was rolled (roll gap: 0.8 cm), folded into half, rounded for five cycles, and put into an aluminium baking pan. The second dough rest was performed for 40 min under the same conditions as for the first dough rest. Baking took place in a deck oven Type-9110-0082 (Binder, Tuttlingen, Germany) at 230 °C for 18 min.

For the determination of the amylase activity in bread all experiments were carried out in triplicate. The breads were stored for 30 h at 22 °C under vacuum. The breads used for the sugar identification and quantitation were either extracted directly after cooling (2 h) or stored under vacuum for up to 96 h at 22 °C. These baking experiments were carried out in fourfold determination.

Determination of amylase activity

Extraction of α-amylase was performed according to Cornaggia et al. [27]. The amylase preparations or bread (1.0 g dry matter) were extracted each with 10 mL extraction buffer (0.1 mol/L sodium maleate, pH 5.5, 1.0 mg/mL bovine serum albumin, 0.025 mol/L DTT) for 16 h at 22 °C. After centrifugation (10 min; 20 °C; 2500×*g*) and filtration (0.45 µm; cellulose acetate) an aliquot (0.1 mL) of the supernatant was used to determine the α-amylase activity with the Ceralpha Method (Megazyme). The supernatant as well as the α-amylase substrate, containing 4-nitrophenyl-β-D-maltoside and a thermostable β-glucosidase, were preincubated at 40 °C. For the assay, the preincubated supernatant and 0.1 mL of preincubated substrate were mixed and incubated for 60 min at 40 °C. The assay was stopped by adding 1.5 mL of 20% (w/v) tri-sodium phosphate buffer (pH 11). Release of 4-nitrophenyl was measured spectrophotometrically at 400 nm. A solution of 1.5 mL 20% (w/v) tri-sodium phosphate buffer (pH 11), 0.1 mL substrate and 0.1 ml enzyme extract was used as blank. α-Amylase activity was expressed in nkat/g_{dm}. One kat is defined as the amount of 4-nitrophenol in mol released from the substrate non reducing-end blocked 4-nitrophenylmaltoheptaoside in one second under the assay conditions. All enzyme extractions as well as determinations of α-amylase activity were performed with three technical replicates.

Extraction, identification, and quantitation of mono-, di-, and oligosaccharides from wheat flour, dough and bread

Breadcrumb (0.5 g dry matter) was extracted using 4.0 mL of 50% (v/v) methanol while stirring for 30 min at 22 °C. The suspension was centrifuged for 20 min and 20 °C at 3500×*g*. The supernatant was dried using a rotary vacuum concentrator (37 °C, 9 h, 800 Pa) to remove the solvent. The samples were reconstituted in 1.0 mL deionized water and purification of the extracts was performed using strong-cation exchange columns (StrataX-C 33 µm, 200 mg, 3 mL, Phenomenex). The columns were activated with methanol (1 × 3.0 mL), equilibrated and washed with 0.1% (v/v) FA (3 × 3.0 mL). After loading the sample, the sugars were eluted with 0.1% (v/v) FA (2 × 2.0 mL). The eluent was removed using a rotary vacuum concentrator and the sugars redissolved in deionized water.

Mono-, di-, and oligosaccharides were analysed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a Dionex ICS-5000 system (Thermo Fisher Scientific) equipped with a CarboPac PA-100 column (250 × 4 mm) connected to a CarboPac PA-100 guard column (50 × 4 mm). The flow rate was 1.0 mL/min. Solvent A was 1.0 mol/L sodium acetate solution, solvent B 1.0 mol/L sodium hydroxide solution and solvent C: deionized water. Solvent B was set to 15% for the total run time. The gradient was 0 min, 0.5% A; 2 min, 0.5% A; 8 min, 10% A; 18 min, 20% A; 23 min, 20% A; 24 min, 0.5% A; 39 min, 0.5% A. The pulsed amperometric detector was equipped with a gold working electrode operating with a standard quadruple waveform. Data analysis was performed using Chromeleon software 7.2 (Thermo Fisher Scientific). Quantitation of the sugars was performed by means of external calibration with standard solutions. Concentration ranges for the calibration were as follows: glucose and fructose, 0.130–0.004 mg/mL; sucrose and maltose, 0.100–0.003 mg/mL; maltotriose, 0.150–0.005 mg/mL; maltotetraose, 0.130–0.004 mg/mL; maltopentaose, 0.180–0.006 mg/mL; maltohexaose, 0.160–0.005 mg/mL; maltoheptaose, 0.180–0.006 mg/mL; maltooctaose, 0.130–0.004 mg/mL.

Statistical analysis

The sugar content of the bread samples is presented as mean value ± standard deviation. To account for the data dependency within each bread sample and to investigate differences of the sugar contents across time points (2 h, 22 h, 48 h, and 96 h after baking) linear mixed models were used. *P*-values were adjusted for multiple testing across time points by the Bonferroni procedure. Statistical analyses were performed with R statistical software (Vienna, Austria).

Results

Protein identification and composition of the amylase preparations

Both bacterial α-amylase preparations (A) and (B) contained one α-amylase each. In the three fungal preparations (C), (D) and (E) one α-amylase (D9J2M5) and one glucoamylase (EC 3.2.1.3, A0A1S9DG38), both from *A. oryzae*, were identified (Table 2). Other proteins from the enzyme group of glycosylases (EC 3.2) were not identified in the bacterial or fungal preparations. The protein composition of the amylase preparations is shown in Fig. 2. Preparation (A) consisted of 85.0% α-amylase P00692. The amount of α-amylase A0A0N0H9C2 in (B) was 21.1%. Both preparations contained various proteins from the respective organism *B. amyloliquefaciens* (A) *and T. vulgaris* (B) (1.6% (A), 6.1% (B)). Wheat proteins were present additionally to the microbial proteins. They were grouped into gluten proteins and other wheat proteins. Preparation (A) contained 13.4% other wheat proteins, whereas (B) contained 38.7% other wheat proteins and 34.1% gluten proteins.

The composition of the fungal α-amylase preparations (C), (D) and (E) was similar in terms of the protein groups. The relative percentage of the fungal α-amylase D9J2M5 was 73.4% in (C), 53.7% in (D) and 79.4% in (E). Compared to D9J2M5 the percentage of glucoamylase A0A1S9DG38 was low: preparations (C) and (D) contained 0.3%, respectively, and (E) 0.2%. The total amount of other *A. oryzae* proteins was 4.0% for (C), 1.4% for (D) and 1.2% for (E), in which A0A1S9DG38 was included due to the low relative percentage. Other wheat proteins were present with 15.4% in (C), 16.8% in (D) and 9.1% in (E) and gluten proteins with 7.2% in (C), 28.1% in (D) and 10.2% in (E).

Table 2 Amylases identified in the preparations with their UniprotKB accession number, the number of peptides identified per amylase, the corresponding protein coverage and the organism to which the amylases were assigned

Preparation	Protein	Enzyme	No. of peptides	Coverage [%]	Organism
(A)	P00692	α-Amylase	33	72.6	*Bacillus amyloliquefaciens*
(B)	A0A0N0H9C2	α-Amylase	25	53.1	*Thermoactinomyces vulgaris*
(C)	D9J2M5	α-Amylase	27	41.4	*Aspergillus oryzae*
	A0A1S9DG38	Glucoamylase	13	21.7	*Aspergillus oryzae*
(D)	D9J2M5	α-Amylase	26	34.9	*Aspergillus oryzae*
	A0A1S9DG38	Glucoamylase	12	21.6	*Aspergillus oryzae*
(E)	D9J2M5	α-Amylase	29	47.6	*Aspergillus oryzae*
	A0A1S9DG38	Glucoamylase	11	21.9	*Aspergillus oryzae*

All protein scores were > 150

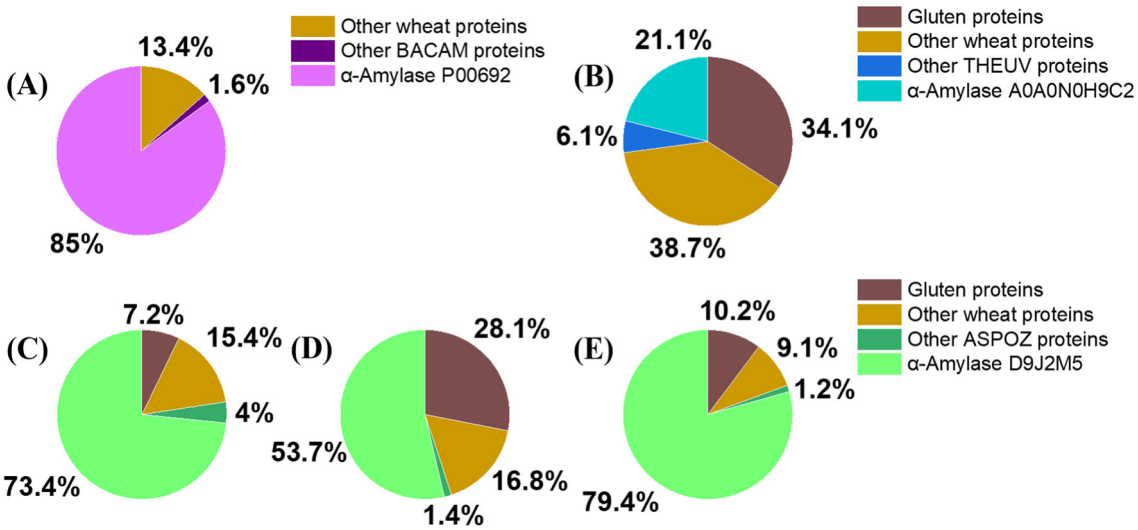

Fig. 2 Relative distribution of wheat and microbial proteins in the tested α-amylase preparations based on MaxQuant iBAQ values. **a** *Bacillus amyloliquefaciens* (BACAM) preparation, **b** *Thermoactinomyces vulgaris* (THEUV) preparation, **c–e** *Aspergillus oryzae* (ASPOZ) preparations

Sugar release during wheat bread storage by endogenous amylase

The sugars glucose, fructose, sucrose, maltose, maltotriose, and maltotetraose were detected in the control breads without any exogenous amylase addition at 2 h, 24 h and 48 h after the end of the baking process (Fig. 3). Neither the qualitative nor the quantitative sugar composition in the bread changed during the first 48 h of storage. The average quantity of total sugar was 27.6 mg/g_{dm} (±4.5), with maltose as the predominant sugar in the breads (14.6 mg/g_{dm} (±1.1) 2 h after baking). Glucose and fructose were present in a similar quantity (glucose: 2.9 mg/g_{dm} (±0.6) and fructose: 2.8 mg/g_{dm} (±2.0)) over 48 h. The average quantity was 5.8 mg/g_{dm} (±0.8) for sucrose, 1.5 mg/g_{dm} (±0.1) for maltotriose and 1.1 mg/g_{dm} (±0.3) for maltotetraose.

Sugar release during wheat bread storage by exogenous amylases

In the breads manufactured with the addition of preparation (A), glucose, fructose, sucrose, maltose and maltotriose were detected 2 h after baking (Fig. 4). Further, maltotetraose was identified 22 h after baking and maltopentaose, maltohexaose, maltoheptaose and maltooctaose were present in the sample 48 h after baking. Two hours after baking 13.1 mg/g_{dm} (±3.0) glucose, 24.8 mg/g_{dm} (±4.6) fructose and 13.8 mg/g_{dm} (±3.5) sucrose were measured in the bread. The total content of mono-, di-, and oligosaccharides was 140.5 mg/g_{dm} (±5.0) 2 h after baking. The amounts of these sugars did not change significantly during the total storage time of 96 h. The maltotriose content increased significantly,

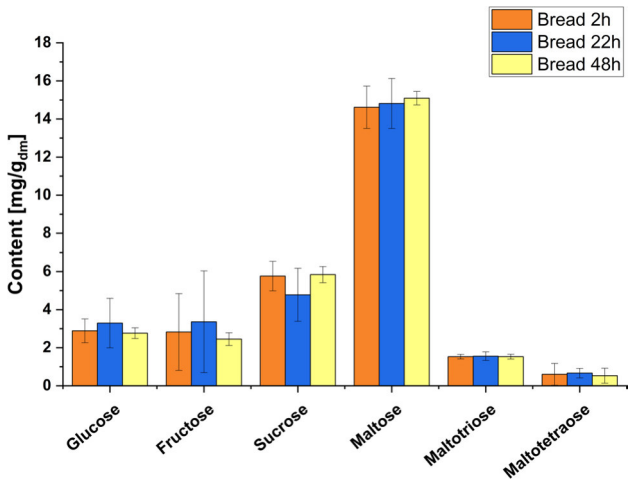

Fig. 3 Sugar content of control breads manufactured without addition of exogenous α-amylase. Samples were taken 2 h, 22 h and 48 h after baking. Values are displayed as means ± standard deviation ($n=4$). There were no significant differences within each sugar between the different time points

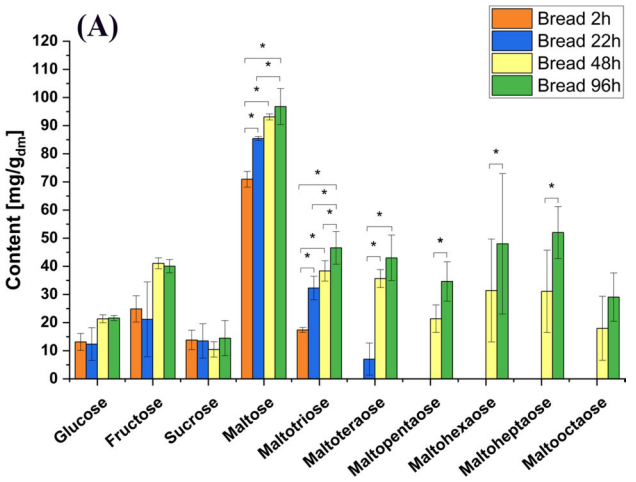

Fig. 4 Sugar content of model breads manufactured with the addition of (A), α-amylase preparation containing α-amylase from *Bacillus amyloliquefaciens*. Samples were taken 2 h, 22 h, 48 h and 96 h after baking. Values are displayed as means ± standard deviation ($n=4$). Significant differences between the different time points for each sugar are indicated by asterisks ($p<0.05$)

starting at 17.4 mg/g_{dm} (±0.9) 2 h after baking to 32.3 mg/g_{dm} (±4.2) 22 h after baking, and from 38.3 mg/g_{dm} (±3.6) 48 h to 46.6 mg/g_{dm} (±5.8) 96 h after baking. Maltotetraose increased significantly from 6.9 mg/g_{dm} (±5.7) 22 h after baking to 43.0 mg/g_{dm} (±8.1) at 96 h. Furthermore, the contents of the sugars identified and quantitated for the first time at 48 h of storage increased significantly until 96 h of storage: Maltopentaose increased from 21.4 mg/g_{dm} (±4.9) to 34.6 mg/g_{dm} (±7.0), maltohexaose from 31.40 mg/g_{dm} (±18.3) to 48.0 mg/g_{dm} (±25.0), maltoheptaose from 31.1 mg/g_{dm} (±14.6) to 52.0 mg/g_{dm} (±9.2). The increase of maltooctaose from 17.9 mg/g_{dm} (±11.4) to 29.1 mg/g_{dm} (±8.6) was not significant.

In the breads manufactured with (B), the composition of the sugar spectrum did not change significantly during the first 48 h of storage (Fig. 5). The total sugar content at 2 h was 44.4 mg/g_{dm} (±3.0). The glucose content of the breads increased significantly from 4.2 mg/g_{dm} (±0.5) at 2 h of storage to 5.6 mg/g_{dm} (±1.1) at 48 h of storage. Further, fructose increased significantly in the first 48 h of storage from 9.5 mg/g_{dm} (±1.2) to 12.4 mg/g_{dm} (±2.5) and maltose increased from 20.3 mg/g_{dm} (±1.2) to 24.7 mg/g_{dm} (±1.3). Between the time points 22 h and 48 h of storage no significant changes were detected for all sugars.

For the three fungal α-amylase preparations (Fig. 6) glucose, fructose, sucrose, maltose, and maltotriose could be identified in the breads. Except for (D), maltotetraose was identified. In general, no significant changes in sucrose were identified for all three preparations. Further, the use of the preparation (C) did not result in significant changes in the contents of glucose, fructose, maltose, and maltotriose. With

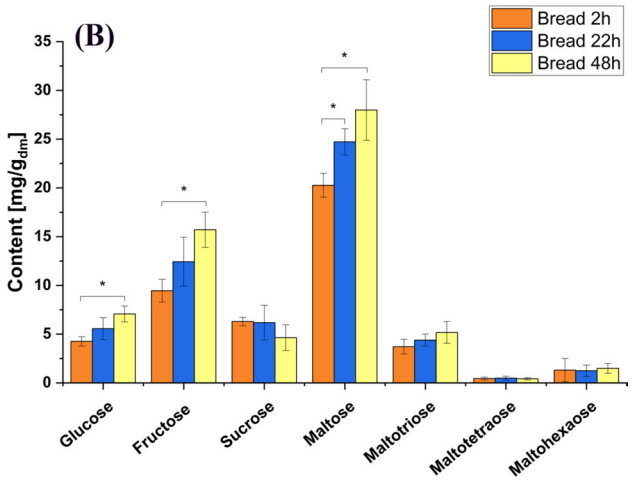

Fig. 5 Sugar content of model breads manufactured with the addition of (B), α-amylase preparation containing α-amylase from *Thermoactinomyces vulgaris.* Samples were taken 2 h, 22 h, 48 h, and 96 h after baking. Values are displayed as means ± standard deviation ($n = 4$). Significant differences between the different time points for each sugar are indicated by asterisks ($p < 0.05$)

the application of (D) no maltotetraose was formed during bread processing. For preparation (D), significant increases were monitored during the 96 h of storage in glucose, fructose, and maltose contents. Glucose increased from 7.8 mg/g$_{dm}$ (± 0.4) at 2 h of storage to 11.4 mg/g$_{dm}$ (± 1.0) at 96 h of storage, fructose increased from 15.0 mg/g$_{dm}$ (± 1.2) at 2 h to 21 mg/g$_{dm}$ (± 1.8) at 96 h and maltose as the predominant sugar increased from 26.5 mg/g$_{dm}$ (± 1.3) to 37.9 mg/g$_{dm}$ (± 1.9) at 96 h. The average total sugar content was 91.0 mg/g$_{dm}$ (± 7.0) for (C) and 55.0 mg/g$_{dm}$ (± 2.2) for (D) 2 h after the end of the baking process.

The application of preparation (E) led to an amount of total sugar in bread of 92.8 mg/g$_{dm}$ (± 3.0) and showed similar changes as (D). The amount of glucose present in the bread manufactured with (E) at 2 h increased significantly from 5.4 mg/g$_{dm}$ (± 0.7) to 23.5 mg/g$_{dm}$ (± 1.4) at 96 h of storage. Fructose increased from 9.1 mg/g$_{dm}$ (± 1.9) at 2 h to 37.3 mg/g$_{dm}$ (± 1.6) at 96 h; maltose increased from 52.2 mg/g$_{dm}$ (± 1.7) to 85.2 mg/g$_{dm}$ (± 2.6) in the same time period.

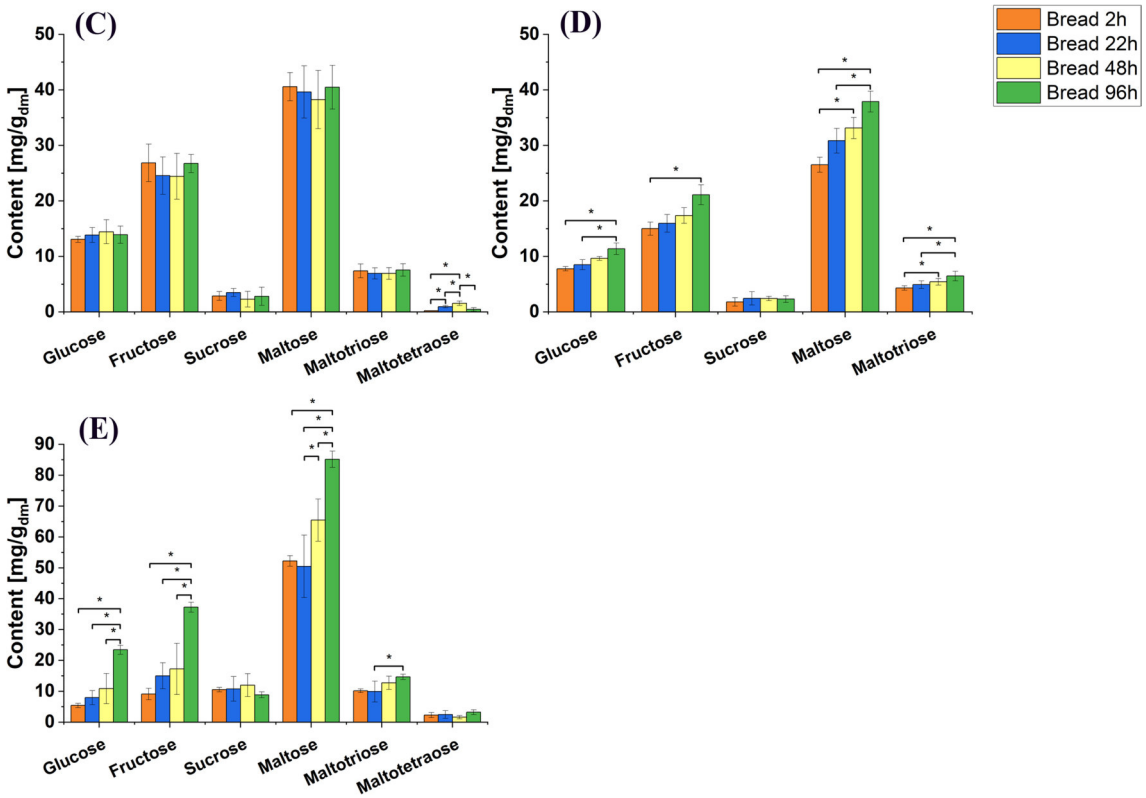

Fig. 6 Sugar content of model breads manufactured with the addition of fungal α-amylase preparations (C), (D) and (E) containing α-amylase from *Aspergillus oryzae.* Samples were taken 2 h, 22 h, 48 h, and 96 h after baking. Significant differences between the different time points for each sugar are indicated by asterisks ($p < 0.05$)

Residual activity of α-amylases in model toast bread

In the wheat flour used for the baking experiments, the activity of endogenous α-amylase was 4.11 nkat/g_{dm} (±0.65). After baking, no activity of endogenous α-amylase was detected in the crumb of the control breads.

The activity of exogenous α-amylases applied in the baking experiments was based on the dosage recommendations of the manufacturers of the preparations. The activity applied to the dough (Table 1) was 1.77 nkat/g_{dm} (±0.03) for preparation (A), 62.74 nkat/g_{dm} (±2.40) for preparation (B), 6.52 nkat/g_{dm} (±1.67) for (C), 77.39 nkat/g_{dm} (±20.83) for (D) and 27.08 nkat/g_{dm} (±1.64) for (E).

For preparation (B) and the fungal preparations (C), (D) and (E) no activity in the crumb of the model toast breads was detected after baking. For preparation (A) an activity of 0.07 nkat/g_{dm} (±0.02) was detected. This corresponded to residual activity of 4.01% in relation to the activity applied to the dough under the selected parameters of dough production and baking.

Discussion

In the present study, we applied a discovery-driven proteomics strategy combined with monitoring of enzymatic hydrolysis products and the quantitation of amylase residual activity in bread to get new insights into the complexity of enzymatic mechanisms in toast bread manufacturing.

The approach using discovery-driven proteomics underlined that amylase preparations used in baking generally contain a small number of functional proteins responsible for the hydrolytic activity of the preparation. This was also shown before by Picariello et al. and Prandi et al. [28, 29]. The iBAQ evaluation is suitable for the relative quantitation of several different proteins in one sample [25, 30] and it showed differences in the protein composition between the preparations. The proportion of α-amylase in the bacterial preparations (A) and (B) was 21.1% and 85.0%, respectively. In preparation (B) gluten proteins and other wheat proteins were detected, indicating that wheat flour was used as filler material. Only non-gluten proteins were present in preparation (A). In particular, the protein P27736, which is a starch synthase and present in wheat starch, shows that (A) contained wheat starch as filler material [31]. The fungal preparations had a similar composition and besides 53.7–79.4% of α-amylase, the other ingredients were wheat flour and various *Aspergillus* proteins.

The baking experiments were carried out without yeast fermentation in a simplified model bread because we intended to focus on differentiating the action of endogenous wheat amylases from exogenous microbial amylases and study the specific changes caused by each of these preparations. Adding yeast with its associated diverse enzymatic activity would have added a potential confounding factor. Due to their sensitivity to heat, the yeast enzymes are unlikely to influence the sugar composition during storage, rather during dough preparation and the early stages of baking. Further experiments would be necessary to study the exact influence of using exogenous microbial amylases in yeast-leavened breads and see how the sugar composition is affected in general. A novel approach using a gas-free crumb pellet system already demonstrated that firming kinetics appeared to be unaffected by the leavening method [32].

The control breads showed no significant changes in the sugar profile during the first 48 h storage. In accordance with Struyf et al. [7], amylase addition in wheat bread-making had a major impact on the maltose levels of dough and bread. Maltose was the sugar found in the highest concentrations in the control breads as well as in the breads with bacterial or fungal α-amylase. For all preparations, the amounts of maltose in bread were higher than in the control bread. Preparation (A) led to an increase in maltose of 385.6% compared to the control breads, which was the largest impact on the maltose content in this study. The maltose content of the breads stored for 2 h and baked with the addition of (B) showed the smallest increase (39.0%) compared to the control bread. All fungal preparations showed different changes in maltose content, which was about 80.1–257.5% higher in the breads with fungal amylase addition compared to the control bread. This could imply that all amylases had at least some exo-activity, but an increased maltose content can also be caused by endo-amylase activity because more non-reducing ends are generated that are susceptible to subsequent β-amylase attack [6].

Two hours after baking a total sugar content between 44.4 mg/g_{dm} (±3.0) for (B) and 140.5 mg/g_{dm} (±5.0) for (A) was observed. Hence, the impact of the preparation on the sugar content is highly dependent on the amylase applied. However, considering the impact of the three fungal preparations on the total sugar content of the breads, we showed that the effect of the same protein could be different: The total sugar content was 91.0 mg/g_{dm} (±7.0) for (C), 54.9 mg/g_{dm} (±2.22) for (D) and 92.8 mg/g_{dm} (±3.0) for (E). There was no correlation between the applied α-amylase activity and the magnitude of change of the sugar spectrum between the breads.

The main difference between the bacterial preparations (A) and (B) regarding the sugar spectrum was the occurrence of new oligosaccharides: maltopentaose, maltohexaose, maltoheptaose and maltooctaose were detected for the first time at 48 h of storage. In addition, a significant increase of 167.8% in maltotriose content between 2 and 96 h after baking was observed (17.4 mg/g_{dm} to 46.6 mg/g_{dm}). Maltotetraose was detected after 22 h of storage with 7.0 mg/g_{dm} and its content increased by 514.3% to 43.0 mg/g_{dm} after

96 h of storage. These maltooligosaccharides, which were formed during storage in considerable amounts, are typical products of α-amylase activity [33]. The effect of new sugars occurring after 48 h of storage was not observed for preparation (B). Furthermore, no significant changes in maltotriose and maltotetraose were observed during the 48 h of storage. For (A), a residual activity of 0.07 nkat/g_{dm}, corresponding to 4.01% of the applied activity of 1.77 nkat/g_{dm}, was determined in the assay, as expected based on previous reports on its temperature stability [34]. By checking the signal-to-noise ratio of the assay, the extinction value corresponding to the residual activity of 0.07 nkat/g_{dm} was proven to be valid. There was no direct relation between the amount of sugar produced during storage and the residual activity [nkat], defined as mol substrate turnover per second. Enzymatic activity in bread is a function of many factors in the enzyme environment including but not limited to the concentration of the substrate, the concentration of the product, the mobility of the enzyme or substrate, the presence of inhibitors, the pH value, and the temperature. However, the temperature optimum of α-amylase from *B. amyloliquefaciens* is 70 °C and it still shows activity at 90 °C [34]. Thus, the changes in maltotriose and maltotetraose as well as the release of new sugars after 48 h can be explained by enzymatic activity. Further research is required to investigate the stability of this enzyme in the bread matrix.

Both preparations (A) and (B) showed an increase in maltose, which was significant during the first 48 h of storage. For (A), it was 22.2 mg/g_{dm} corresponding to 31.3% and for (B), it was 7.7 mg/g_{dm} or 37.9%. Due to the lack of residual activity for the *T. vulgaris* α-amylase in preparation (B) this effect cannot be caused by residual activity. The changes in maltose content may be related to changes in the extractability of sugars from bread due to starch retrogradation. Lin and Lineback [35] also showed that the amount of extractable maltooligosaccharides in wheat bread without and with the addition of α-amylase changed during 6 days of storage, but they did not propose a mechanism on a molecular level. More recently, several mechanisms on how mono-, di- and oligosaccharides influence starch gelatinization have been discussed based on water activity, glass transition temperatures, and intermolecular hydrogen bonding [36]. The denaturation and melting behavior of biopolymers, like starch, in the presence of plasticizers such as sugars and polyols was found to be related to the effective molar density of hydroxyl groups available for intermolecular hydrogen bonding, also designated as effective solvent volume fraction [37]. This term was found to describe gelatinization in ternary starch/sugar/water mixtures and it was linked to the volumetric density of intermolecular hydrogen bonds [38]. Therefore, it might be possible that hydrogen bonds between starch and sugars are weakened during retrogradation and lead to a change in the extractability of sugars over time. Because the overall sugar contents in the control bread were considerably lower than in the breads with enzyme addition, the changes in extractability are expected to be smaller.

All fungal α-amylase preparations (C), (D) and (E) showed no residual activity in the assays performed. Fungal α-amylase showed a lower inactivation temperature than bacterial α-amylase at about 80 °C [16]. Despite the irreversible inactivation of the α-amylase from *A. oryzae* in preparations (C), (D) and (E), significant changes in the sugar spectrum of the breads manufactured with the preparations (D) and (E) were observed. In the breads manufactured with the respective preparation, glucose, fructose, and maltose content significantly increased during the 96 h of storage. It may be possible that the change in the sugar spectrum without a measurable residual amylase activity is related to changes in the extractability of sugars caused by starch retrogradation [35].

Conclusion

Five different α-amylase preparations commonly applied in bread production were analyzed regarding their protein composition and their impact on straight dough wheat breads. We identified three α-amylases of different origins in the sample set. Preparation (A) with α-amylase from *B. amyloliquefaciens* showed the largest impact on the total sugar content of bread, with an increase of 408.6% compared to the control (at 2 h). The fungal preparations did not show a uniform impact on the total sugar content with increases ranging from 99.0 to 229.6% compared to the control. Residual activity of 0.07 nkat/g_{dm} corresponding to 4.01% of the applied activity was determined in the breads of preparation (A). This resulted in a significant increase of maltotriose and maltotetraose during storage as well as the formation of new sugars, but we cannot conclude whether the residual activity measured after extraction from the crumb of baked bread has any technological function on the bread during storage. Our proteomics-based approach coupled with the quantitation of sugars and the detection of residual activity in bread allowed observing the activity of exogenous enzymes in straight dough wheat bread during storage for the first time.

Acknowledgements The authors thank Stefanie Hackl and Angelika Grassl for excellent technical assistance, Dr. Sabrina Geisslitz (KIT) for helpful discussions, and Dr. Leonie Weinhold (IMBIE, University Hospital Bonn) for advice on statistical evaluation.

Author contributions Conceptualization: GFR, KAS; methodology: GFR, KAS; formal analysis, investigation: GFR, KS, SD; writing—original draft, visualization: GFR; writing—review and editing: KS, SD, CD, TH, KAS; resources: CD, TH; supervision, project administration: KAS.

Funding Open Access funding enabled and organized by Projekt DEAL. This IGF Project of the FEI was supported via AiF within the programme for promoting the Industrial Collective Research (IGF) of the German Ministry of Economic Affairs and Energy (BMWi), based on a resolution of the German Parliament. Project AiF 19543N.

Compliance with ethical standards

Conflict of interest The authors declare no competing financial interest.

Human and animal rights statement This article does not contain any studies with human or animal subjects.

References

1. Dupont FM, Altenbach SB (2003) Molecular and biochemical impacts of environmental factors on wheat grain development and protein synthesis. J Cereal Sci 38:133–146. https://doi.org/10.1016/S0733-5210(03)00030-4
2. Whitehurst RJ, van Oort M (eds) (2010) Enzymes in food technology. Wiley-Blackwell, Chichester, pp 109–111
3. Bosmans GM, Lagrain B, Fierens E, Delcour JA (2013) Impact of amylases on biopolymer dynamics during storage of straight-dough wheat bread. J Agric Food Chem 61:6525–6532. https://doi.org/10.1021/jf402021g
4. Jiménez T, Martínez-Anaya MA (2001) Amylases and hemicellulases in breadmaking. Degradation by-products and potential relationship with functionality. Food Sci Technol Int 7:5–14. https://doi.org/10.1106/7U5G-5AKQ-HVBQ-4XA0
5. Tebben L, Shen Y, Li Y (2018) Improvers and functional ingredients in whole wheat bread: a review of their effects on dough properties and bread quality. Trends Food Sci Technol 81:10–24. https://doi.org/10.1016/j.tifs.2018.08.015
6. Struyf N, Verspreet J, Courtin CM (2016) The effect of amylolytic activity and substrate availability on sugar release in non-yeasted dough. J Cereal Sci 69:111–118. https://doi.org/10.1016/j.jcs.2016.02.016
7. Struyf N, Verspreet J, Verstrepen KJ, Courtin CM (2017) Investigating the impact of α-amylase, α-glucosidase and glucoamylase action on yeast-mediated bread dough fermentation and bread sugar levels. J Cereal Sci 75:35–44. https://doi.org/10.1016/j.jcs.2017.03.013
8. Cho IH, Peterson DG (2010) Chemistry of bread aroma: a review. Food Sci Biotechnol 19:575–582. https://doi.org/10.1007/s10068-010-0081-3
9. Hug-Iten S, Fischer F, Conde-Petit B (2001) Structural properties of starch in bread and bread model systems: Influence of an antistaling alpha-amylase. Cereal Chem 78:421–428
10. Palacios HR, Schwarz PB, D'Appolonia BL (2004) Effect of alpha-amylases from different sources on the retrogradation and recrystallization of concentrated wheat starch gels: relationship to bread staling. J Agric Food Chem 52:5978–5986. https://doi.org/10.1021/jf030377z
11. Defloor I, Delcour JA (1999) Impact of maltodextrins and antistaling enzymes on the differential scanning calorimetry staling endotherm of baked bread doughs. J Agric Food Chem 47:737–741. https://doi.org/10.1021/jf9806356
12. Goesaert H, Leman P, Bijttebier A, Delcour JA (2009) Antifirming effects of starch degrading enzymes in bread crumb. J Agric Food Chem 57:2346–2355. https://doi.org/10.1021/jf803058v
13. Perdana J, Fox MB, Schutyser MAI, Boom RM (2012) Enzyme inactivation kinetics: coupled effects of temperature and moisture content. Food Chem 133:116–123. https://doi.org/10.1016/j.foodchem.2011.12.080
14. Zhang L, Chen XD, Boom RM, Schutyser MAI (2017) Thermal inactivation kinetics of β-galactosidase during bread baking. Food Chem 225:107–113. https://doi.org/10.1016/j.foodchem.2017.01.010
15. Lagrain B, Leman P, Goesaert H, Delcour JA (2008) Impact of thermostable amylases during bread making on wheat bread crumb structure and texture. Food Res Int 41:819–827. https://doi.org/10.1016/j.foodres.2008.07.006
16. Cauvain S (2015) Technology of breadmaking. Springer International Publishing, Cham
17. Reichenberger K, Luz A, Seitl I, Fischer L (2020) Determination of the direct activity of the maltogenic amylase from *Geobacillus stearothermophilus* in white bread. Food Anal Methods 13:496–502. https://doi.org/10.1007/s12161-019-01673-7
18. Regulation (EC) (2008) No 1332/2008 of the European Parliament and of the Council of 16 December 2008on food enzymes and amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/9
19. Regulation (EU) (2011) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/200
20. Uhr L, Buchholz T, Homann T, Huschek G (2014) Targeted proteomics-based analysis of technical enzymes from fungal origin in baked products. J Cereal Sci 60:440–447. https://doi.org/10.1016/j.jcs.2014.04.007
21. Schalk K, Lexhaller B, Koehler P, Scherf KA (2017) Isolation and characterization of gluten protein types from wheat, rye, barley and oats for use as reference materials. PLoS ONE 12:e0172819. https://doi.org/10.1371/journal.pone.0172819
22. Van Eckert R, Berghofer E, Ciclitira PJ, Chirdo F, Denery-Papini S, Ellis HJ, Ferranti P, Goodwin P, Immer U, Mamone G, Méndez E, Mothes T, Novalin S, Osman A, Rumbo M, Stern M, Thorell L, Whim A, Wieser H (2006) Towards a new gliadin reference material–isolation and characterisation. J Cereal Sci 43:331–341. https://doi.org/10.1016/j.jcs.2005.12.009
23. European Food Safety Authority, Parma, IT (2019) https://www.efsa.europa.eu/de/search/site/amylase. Accessed 1 Jul 2019
24. Food and Drug Administration, Silver Spring, USA (2019) https://search.usa.gov/search?query=amylase&affiliate=fda1. Accessed 1 Jul 2019

25. Schwanhäusser B, Busse D, Li N, Dittmas G, Schuchhardt J, Wolf J, Chen W, Selbach M (2011) Global quantification of mammalian gene expression control. Nature 473:337–342. https://doi.org/10.1038/nature10098
26. Kieffer R, Wieser H, Henderson MH, Graveland A (1998) Correlations of the breadmaking performance of wheat flour with rheological measurements on a micro-scale. J Cereal Sci 27:53–60. https://doi.org/10.1006/jcrs.1997.0136
27. Cornaggia C, Evans DE, Draga A, Mangan D, McCleary BV (2019) Prediction of potential malt extract and beer filterability using conventional and novel malt assays. J Inst Brew 125:294–309. https://doi.org/10.1002/jib.567
28. Picariello G, Di Stasio L, Mamone G, Iacomino G, Venezia A, Iannaccone N, Ferranti P, Coppola R, Addeo F (2018) Identification of enzyme origin in dough improvers: DNA-based and proteomic approaches. Food Res Int 105:52–58. https://doi.org/10.1016/j.foodres.2017.10.046
29. Prandi B, Lambertini F, Varani M, Faccini A, Suman M, Leorati A, Tedeschi T, Sforza S (2020) Assessment of enzymatic improvers in flours using LC-MS/MS detection of marker tryptic peptides. J Am Soc Mass Spectrom 31:240–248. https://doi.org/10.1021/jasms.9b00109
30. Geisslitz S, Ludwig C, Scherf KA, Koehler P (2018) Targeted LC-MS/MS reveals similar contents of α-amylase/trypsin-inhibitors as putative triggers of nonceliac gluten sensitivity in all wheat species except einkorn. J Agric Food Chem 66:12395–12403. https://doi.org/10.1021/acs.jafc.8b04411
31. Ainsworth C, Clark J, Balsdon J (1993) Expression, organisation and structure of the genes encoding the waxy protein (granule-bound starch synthase) in wheat. Plant Mol Biol 22:67–82
32. Paulik S, Jekle M (2019) Novel approach to investigate the mechanical properties of crumb matrix during storage—re-engineering of gas-free crumb pellets. Food Chem 288:333–340. https://doi.org/10.1016/j.foodchem.2019.03.014
33. van der Maarel MJEC, van der Veen B, Uitdehaag JCM, Leemhuis H, Dijkhuizen L (2002) Properties and applications of starch-converting enzymes of the α-amylase family. J Biotechnol 94:137–155. https://doi.org/10.1016/S0168-1656(01)00407-2
34. Brzozowski AM, Lawson DM, Turkenburg JP, Bisgaard-Frantzen H, Svendsen A, Borchert TV, Dauter Z, Wilson KS, Davies GJ (2000) Structural analysis of a chimeric bacterial alpha-amylase. High-resolution analysis of native and ligand complexes. Biochemistry 39:9099–9107. https://doi.org/10.1021/bi0000317
35. Lin W, Lineback DR (1990) Changes in carbohydrate fractions in enzyme-supplemented bread and the potential relationship to staling. Starke 42:385–394
36. Allan MC, Rajwa B, Mauer LJ (2018) Effects of sugars and sugar alcohols on the gelatinization temperature of wheat starch. Food Hydrocoll 84:593–607. https://doi.org/10.1016/j.foodhyd.2018.06.035
37. Van der Sman RGM (2016) Sugar and polyol solutions as effective solvent for biopolymers. Food Hydrocoll 56:144–149. https://doi.org/10.1016/j.foodhyd.2015.12.001
38. Van der Sman RGM, Mauer LJ (2019) Starch gelatinization temperature in sugar and polyol solutions explained by hydrogen bond density. Food Hydrocoll 94:371–380. https://doi.org/10.1016/j.foodhyd.2019.03.034

3.2 Impact of exogenous maltogenic α-amylase and maltotetraogenic amylase on sugar release on white bread

Five different commercially available maltogenic α-amylase preparations and two maltotetraogenic amylase preparations were investigated regarding their protein composition. Using baking experiments, the residual activity of amylases in the bread crumb and the effects of the preparations on the sugar composition of the bread crumb were analyzed. Gerold Rebholz planned and performed the experiments, and collected, analyzed, and interpreted the data.

Via discovery-driven proteomics the amylases which caused the activity of the respective preparations were identified, and their share within the proteins present was quantitated by intensity based absolute quantitation (iBAQ). A residual activity in bread crumb was measured for all amylases included in the study. The relative activity compared to the applied activity was 1.1% - 1.9% for the maltotetraogenic amylases and 13.9% - 45.5% for the maltogenic α-amylases. This confirms the high thermal stability of the maltogenic α-amylase used in wheat bread baking. Moreover, the changes in sugar spectrum during storage were quite different. This includes the absolute amount of sugar formed during storage as well as the composition of the individual sugar spectrum. Whether the detected residual activity is related to the characteristics of bread staling or bread crumb properties must be clarified in further studies.

Based on our work, we were able to describe the link between the applied amylases, their residual activity and the resulting changes in the sugar spectrum of wheat bread crumb. This provides the basis for further research regarding a possible technological impact of maltotetraogenic amylase and maltogenic α-amylase in wheat bread.

In addition to the practical research work Gerold Rebholz wrote the manuscript, designed the figures and tables and revised it according to the comments of the co-authors and reviewers.

European Food Research and Technology
https://doi.org/10.1007/s00217-021-03721-1

ORIGINAL PAPER

Impact of exogenous maltogenic α-amylase and maltotetraogenic amylase on sugar release in wheat bread

Gerold Felix Rebholz[1] · Karin Sebald[2] · Sebastian Dirndorfer[2] · Corinna Dawid[2,3] · Thomas Hofmann[2] · Katharina Anne Scherf[1,4]

Received: 7 January 2021 / Revised: 9 March 2021 / Accepted: 12 March 2021

Abstract

The use of exogenous maltogenic α-amylases or maltotetraogenic amylases of bacterial origin is common in wheat bread production, mainly as antistaling agents to retard crumb firming. To study the impact of maltogenic α-amylase and maltotetraogenic amylase on straight dough wheat bread, we performed a discovery-driven proteomics approach with commercial enzyme preparations and identified the maltotetraogenic amylase P22963 from *Pelomonas saccharophila* and the maltogenic α-amylase P19531 from *Geobacillus stearothermophilus*, respectively, as being responsible for the amylolytic activity. Quantitation of mono-, di- and oligosaccharides and residual amylase activity in bread crumb during storage for up to 96 h clarified the different effects of residual amylase activity on the sugar composition. Compared to the control, the application of maltogenic α-amylase led to an increased content of maltose and especially higher maltooligosaccharides during storage. Residual amylase activity was detectable in the breads containing maltogenic α-amylase, whereas maltotetraogenic amylase only had a very low residual activity. Despite the residual amylase activities and changes in sugar composition detected in bread crumb, our results do not allow a definite evaluation of a potential technological function in the final product. Rather, our study contributes to a fundamental understanding of the relation between the specific amylases applied, their residual activity and the resulting changes in the saccharide composition of wheat bread during storage.

Keywords Bread · Discovery-driven proteomics · Enzyme activity · Maltogenic α-amylase · Maltotetraogenic amylase · Wheat

Introduction

Wheat bread can be considered as an unstable multiphase food matrix subject to changes during storage: the flavor of fresh bread and the crispiness of the bread crust decrease, whereas crumb firmness and crumbliness increase. The changes in the bread crumb, also known as staling, lead to a loss of resilience and to firming [1], caused by water immobilization and redistribution processes. The retrogradation of amylopectin during storage immobilizes water, which leads to a decrease in freezable water in the bread crumb [2]. Furthermore, water redistribution occurs via water migration from gluten to starch and from crumb to crust. These migration patterns lead to an additional decrease in freezable water and to an increase in crumb firmness [3, 4]. Wheat starch rearrangements play the leading role in crumb firming. Amylose gelation occurs within hours, whereas that of amylopectin takes several days and involves reorganization of amylopectin side chains to form tightly

✉ Katharina Anne Scherf
katharina.scherf@kit.edu

1 Leibniz-Institute for Food Systems Biology at the Technical University of Munich, Lise-Meitner-Str. 34, 85354 Freising, Germany

2 Chair of Food Chemistry and Molecular Sensory Science, TUM School of Life Sciences, Technical University of Munich, Lise-Meitner-Str. 34, 85354 Freising, Germany

3 Bavarian Center for Biomolecular Mass Spectrometry, Technical University of Munich, Gregor-Mendel-Str. 4, 85354 Freising, Germany

4 Department of Bioactive and Functional Food Chemistry, Institute of Applied Biosciences, Karlsruhe Institute of Technology (KIT), Adenauerring 20 a, 76131 Karlsruhe, Germany

Published online: 26 March 2021

packed double-helical structures. During storage, the amorphous gelatinized starch network present in fresh bread is transformed first into a partly crystalline state and then to a mostly continuous rigid crystalline starch network [3, 5, 6].

Amylases can be added during bread production as antistaling agents [7]. Maltotetraogenic amylases and maltogenic α-amylases are most commonly described as efficient antistaling agents in bread making [6, 8]. Maltotetraogenic amylase from *Pelomonas saccharophila* hydrolyzes α-(1 → 4)-D-glucosidic bonds to remove successive maltotetraose residues from the non-reducing ends. It mainly acts on amylopectin, thus reducing amylopectin side chain rearrangement and crystallization [9, 10]. Maltogenic α-amylase from *Geobacillus stearothermophilus* successively cleaves α-maltose units from the non-reducing ends of amylose and amylopectin. In comparison to maltotetraogenic amylase, it has a smaller effect on the molecular weight of the starch polymers and degrades the amylopectin molecules relatively slowly [8, 10]. However, the enzyme reduces the molecular weight of amylose and thus, enhances amylose mobility and network formation [5]. This results in a greater initial firmness of breads manufactured with maltogenic α-amylase [11, 12]. The main influence on bread firming caused by maltogenic α-amylase is due its subsequent cleavage along the side chains of the amylopectin molecules. They become too short to crystallize and the formation of a permanent amylopectin network during storage is inhibited [8, 10]. Despite the initial increase in crumb firmness, this leads to a substantial delay in crumb firming [12].

An additional antistaling effect of maltotetraogenic and maltogenic α-amylases may result from their ability to increase dextrin levels in wheat bread. Dextrins hinder the crystallization of amylopectin helices and may increase relative crumb humidity. However, compared to the hydrolytic effect on the starch polymers, the antistaling effect of dextrins is described to be of minor importance [13]. Maltotetraogenic amylase from *P. saccharophila* and maltogenic α-amylase from *G. stearothermophilus* have a temperature optimum at approximately 60 °C [14]. Several studies showed that both enzymes have a high temperature stability, whereby it is accepted that *G. stearothermophilus* maltogenic α-amylase has a higher temperature stability and can be still active in wheat bread crumb after baking [8, 15, 16]. It is unknown whether this activity is associated with an additional antistaling effect during storage of bread. In the following, residual activity means the amylolytic activity measured using enzymatic assays after extraction from baked bread crumb. To gain a better understanding of the effects of maltogenic α-amylase and maltotetraogenic amylase in wheat bread making, it is necessary to study the formulation of the specific preparations as well as the impact of the preparations on sugar release during bread storage. Thus, a combined analytical approach has to be implemented to focus on the different relevant parameters in amylase preparations and wheat bread.

The use of enzymes in food production is defined in the European Union by the Regulation (EC) no. 1332/2008 and has to be in line with the Regulation (EU) no. 1169/2011 on the provision of food information to consumers [17, 18]. According to these regulations, enzymes used as processing aids do not have to be labeled, because they are not classified as food ingredients of technological relevance in the final product. However, only in case this potential residual activity would have a functional effect in the final product, it would have to be labeled [17, 18].

The aim of our work was to characterize the protein composition of commercial amylase preparations commonly used in bread making and to elucidate their impact on the formation of sugars in the bread crumb during storage. In the first step, we identified and quantitated the amylase compared to other proteins in the preparation. Then we monitored sugar concentrations in wheat bread crumb during storage for up to 96 h and combined this with the quantitation of residual amylase activity. This approach allowed us to detect changes caused by a residual amylolytic activity in straight dough wheat bread.

Materials and methods

Chemicals and flours

All chemicals and solvents were HPLC-grade or higher. Water was deionized and purified using an Arium 611VF water purification system (Sartorius, Goettingen, Germany). Trypsin (from bovine pancreas, TPCK-treated, ≥ 10,000 BAEE U/mg protein), sodium hydroxide solution (50–52%; eluent for IC) and anhydrous sodium acetate (≥ 99%) were from Sigma-Aldrich (Steinheim, Germany). α-Amylase assay reagent (4-nitrophenyl α-D-maltoheptaoside (blocked), plus excess α-glucosidase and glucoamylase) and β-amylase assay reagent (4-nitrophenyl β-D-maltotrioside, plus excess thermostable β-glucosidase) were purchased from Megazyme (Bray, Ireland). D-(+)-Glucose (≥ 99.5%), D-(-)-fructose (≥ 99%), sucrose (≥ 99.5%), maltose monohydrate (≥ 99%), maltotriose (≥ 90%) and maltoheptaose (≥ 70%) were from Sigma-Aldrich; maltotetraose (≥ 97%), maltopentaose (≥ 90%), maltohexaose (≥ 95%) and maltooctaose (≥ 80%) from Santa Cruz (Dallas, USA). Wheat flour type 550, according to the German flour classification system, was provided by Rosenmühle (Ergolding, Germany).

Amylase preparations

Seven different commercial amylase preparations commonly applied in wheat bread making were used (Table 1). Two of them, (I) and (II), contained maltotetraogenic amylase (EC 3.2.1.60) from *P. saccharophila.* The maltogenic α-amylase (EC 3.2.1.133) in the preparations (III), (IV), (V), (VI), and (VII) was from *G. stearothermophilus*.

Determination of maltotetraogenic amylase activity

The extraction of amylases for the activity assays was performed according to Cornaggia et al. [19]. In total, 1.0 g of dry matter of amylase preparation or bread was extracted with 10 mL of extraction buffer (0.1 mol/L sodium maleate, pH 5.5, 1.0 mg/mL bovine serum albumin, 0.025 mol/L dithiothreitol (DTT)) for 16 h at 22 °C. Then, centrifugation (2500×*g*; 20 °C; 10 min) and filtration (0.45 µm; cellulose acetate filters) were performed.

The activity of maltotetraogenic amylase was determined using the Ceralpha method (Megazyme). The assay procedure was carried out as described by the manufacturer, except that the incubation time was extended to 60 min at 40 °C for the assay reaction. Quantitation of 4-nitrophenol was conducted by means of external calibration with a standard solution containing 4-nitrophenol dissolved and diluted with 1.5 mL of 20% (w/v) tri-sodium phosphate buffer (pH 11). Maltotetraogenic amylase activity was reported in nkat/g_{dm}. One kat is defined as the amount of 4-nitrophenol in mol released from the substrate non reducing-end blocked 4-nitrophenylmaltoheptaoside in one second under the assay conditions. All determinations were performed with three technical replicates.

Determination of maltogenic α-amylase activity

The extraction of maltogenic α-amylase was carried out as described above according to Cornaggia et al. [19]. Then the betamyl-3 method (Megazyme) was used to determine maltogenic α-amylase activity. The amylase extract was diluted in an appropriate ratio with 0.1 mol/L 2-(*N*-morpholino)ethanesulfonic acid buffer (pH 6.2) containing 0.01 mol/L ethylenediaminetetraacetic acid and 1.0 mg/mL bovine serum albumin. For the assay procedure, the incubation time of the extract and the substrate was set to 20 min at 40 °C. The following procedure was performed exactly as described by the kit manufacturer. Quantitation of 4-nitrophenol was performed by means of external calibration with a standard solution containing 4-nitrophenol dissolved and diluted in 1% (w/v) tris(hydroxymethyl)aminomethane (Tris)-buffer (pH 8.5). Amylase activity was expressed in nkat/g_{dm}. One kat is defined as the amount of 4-nitrophenol in mol released from the substrate non reducing-end blocked 4-nitrophenyl-β-D-maltotrioside in 1 s under the assay conditions. All determinations were performed with three technical replicates.

Extraction of enzymes from the amylase preparations and determination of the protein content

For ultra high-performance liquid chromatography time-of-flight mass spectrometry (UHPLC–TOF–MS) analysis, the amylases were extracted from the preparations as described by Uhr et al. [20]. Each sample (0.5 g of dry matter) was extracted using 5.0 mL of extraction buffer (0.1 mol/L ammonium bicarbonate, 4.0 mol/L urea and 0.005 mol/L DTT, pH 8.0, in water). The extraction was carried out at

Table 1 Composition of the bacterial amylase preparations and dosage recommendation as indicated by the manufacturer, activity of the amylase added to the dough in the baking experiments, residual activity in the bread crumb and relative residual activity based on the activity added to the dough

Preparation	Enzyme	EC number	Other ingredients	Dosage (mg/kg of flour)	Activity (nkat/g_{dm})	Residual activity		
						(nkat/g_{dm})	Mean (%)	Range (%)
(I)	Maltotetraogenic amylase	3.2.1.60	Sodium chloride, wheat starch	850	85.8	0.9 ± 0.3	1.1	0.6–1.4
(II)	Maltotetraogenic amylase	3.2.1.60	Sodium chloride, wheat starch, microcrystalline cellulose	300	31.7	0.6 ± 0.2	1.9	1.2–2.9
(III)	Maltogenic α-amylase	3.2.1.133	Wheat flour, sodium chloride	100	22.0	9.3 ± 1.4	42.3	38.0–47.7
(IV)	Maltogenic α-amylase	3.2.1.133	Maltodextrin, sodium chloride	100	43.0	11.0 ± 5.3	25.7	11.3–35.7
(V)	Maltogenic α-amylase	3.2.1.133	Maltodextrin, sodium chloride, sunflower oil	100	38.6	11.0 ± 5.0	28.6	18.5–43.9
(VI)	Maltogenic α-amylase	3.2.1.133	Wheat flour, sodium chloride	100	31.5	14.3 ± 3.8	45.5	36.3–51.0
(VII)	Maltogenic α-amylase	3.2.1.133	Maltodextrin, sodium chloride, sunflower oil	50	41.0	5.7 ± 1.2	13.9	11.9–15.0

dm, based on dry matter, values are given as means ($n = 3$) ± standard deviation

room temperature by stirring for 30 min. The extract was centrifuged (20 min; 20 °C; 3750×*g*) and the supernatant was collected for further analysis. To optimize the conditions for the tryptic digest of extracts, the protein contents were quantified via a RP-HPLC method with UV detection at 210 nm as described earlier [21].

Sample preparation, tryptic digest, and peptide clean-up

The supernatant of the enzyme extracts was diluted in an appropriate volume to a protein concentration of 0.5 mg/mL in 0.3 mL and 0.1 mL Tris–HCl (1.5 mol/L) and 0.1 mL 1-propanol were added. Reduction was performed by adding tris(2-carboxyethyl)phosphine solution (0.05 mol/L tris(2-carboxyethyl)phosphine in 0.5 mol/L Tris–HCl, pH 8.5) and incubation for 30 min at 60 °C. Alkylation of cysteine residues was performed for 45 min at 37 °C in the dark using 0.1 mL chloroacetamide solution (0.5 mol/L chloroacetamide in 0.5 mol/L Tris–HCl, pH 8.5). Then the samples were dried using a rotary vacuum concentrator (37 °C, 3 h, 800 Pa). For protein hydrolysis, the samples were reconstituted in 0.5 mL of trypsin stock solution with a trypsin-to-substrate ratio of 1:20 (w/w) (0.04 mol/L urea in 0.1 mol/L Tris–HCl, pH 7.8) and incubated at 37 °C for 16 h. The hydrolysis was stopped by heating the samples at 95 °C for 8 min. The peptide mixture was cleaned by solid phase extraction (SPE) using 100 mg Discovery DSC-18 tubes (Merck, Darmstadt, Germany). The tubes were activated with methanol (1 mL), equilibrated with acetonitrile/water/formic acid (FA) (80:20:0.1; 1 mL) and washed with acetonitrile/water/FA (2:98:0.1; 5 × 1 mL). Having loaded the samples, the cartridges were washed again (5 × 1 mL). The peptides were eluted with acetonitrile/water/FA (40:60:0.1) and dried using a rotary vacuum concentrator (37 °C, 4 h, 800 Pa). For UHPLC-TOF–MS analysis, the peptides were reconstituted in 500 µL FA and filtered (0.45 µm; wwPTFE).

Discovery driven LC–MS/MS analysis

A Sciex TripleTOF 6600 mass spectrometer (Sciex, Darmstadt, Germany) connected to a Shimadzu Nexera X2 system (Shimadzu, Kyoto, Japan) operating in the positive electrospray ionization (ESI) mode was used for UHPLC–TOF–MS analysis. Control of the instrument and data acquisition were performed using AnalystTF software (version 1.7.1, Sciex). The chromatography system consisted of a 150 × 2.1 mm, 1.6 µm BioZen Peptide PS-C-18 LC column (Phenomenex, Aschaffenburg, Germany) equipped with a security guard ultra-column (2 × 2.1 mm) of the same type. The injection volume was 10 µL and the temperature of the column oven was set to 40 °C. The elution solvents were (A) 1% (v/v) aqueous FA and (B) acetonitrile containing 1% (v/v) FA. A 75 min gradient was used to separate the peptides with a flow rate of 0.35 mL/min starting at 0 min, 5% B; 5 min, 5% B; 60 min, 40% B; 65 min, 100% B; 69 min, 100% B; 70 min, 5% B; and 75 min, 5% B. The following parameters were set: ion spray voltage: 5500 eV, source temperature: 550 °C, nebulizing gas: 55 psi, heating gas: 65 psi and nitrogen as curtain gas: 35 psi.

A TOF–MS survey scan was acquired in the information-dependent acquisition mode (IDA), from *m*/*z* 400 to 1000 using 250 ms as accumulation time, 10 V as collision energy (CE) and 80 V as declustering potential (DP). Product ion spectra were recorded in the high-resolution mode for 70 ms for the 12 most abundant compounds. The *m*/*z* range was *m*/*z* 100–2000 using a DP of 80 V and CE of 40 V, with CE spread of 15 V. IDA criteria were set for the precursor ion intensity at > 100 counts/s. Q1 resolution was set to 0.7 Da.

Identification of amylases using MaxQuant

TOF raw files were used for the identification and relative quantitation of proteins using MaxQuant (version 1.6.10.43) [22]. A workflow with different searches against databases derived from UniProtKB using the search engine Andromeda was developed [23]. The first step of data evaluation comprised the identification of enzymes from the class EC 3.2.-.- (glycosylases). A database with all proteins belonging to the class EC 3.2.-.- from *Triticum aestivum* and from 14 microorganisms relevant for the production of food grade amylases according to EFSA and FDA was built [24, 25]. This database contained 5550 proteins (downloaded from UniProtKB on July 01, 2019). In the second step, all proteins present in the sample were identified, using databases containing the proteome of the identified organisms, respectively. In the final step, the relative quantitation of the proteins was performed using the intensity based absolute quantitation (iBAQ) algorithm implemented within MaxQuant [26]. For each sample, an individual database was built containing all proteins identified in the second step. A total sum normalization of iBAQ protein intensities between samples was performed to correct for the different total protein injection amounts.

The MaxQuant parameters were set as follows for all searches: Variable modifications: oxidation of methionine, N-terminal protein acetylation; fixed modification: carbamidomethylation on cysteine; proteolytic enzyme: trypsin; missed cleavage sites: up to two. Match-between-runs was enabled with a matching time window of 0.7 min and an alignment time window of 20 min. Results were filtered for a minimal length of five, a maximal length of 50 amino acids and 1% peptide and protein false discovery rate.

The content of amylase [mg/g] present in the preparations added in the baking experiments, respectively, was calculated based on the protein contents of the amylase extracts

for UHPLC–TOF–MS analysis considering the percentage of amylase determined via iBAQ. Thus, the absolute protein content in the sample was multiplied with the relative iBAQ value for each amylase.

Baking experiments

The recipe for the baking experiments was: Wheat flour type 550 (50 g, based on 14% moisture), 2.0 g baking soda, 0.25 g sodium chloride and bulk liquid as determined in the Farinograph-E [27]. Water as bulk liquid was replaced by a buffer solution with 0.1 mol/L citric acid monohydrate and 0.1 mol/L trisodium citrate dihydrate (pH 2.75) to obtain a pH comparable to yeast-leavened wheat bread (pH 6). Baking soda was added 1 min before the end of the kneading time. The dough was kneaded at 60 rpm to optimum consistency in the Farinograph-E [27]. After kneading, the dough was rounded for eight cycles and placed in the proofing cabinet for 20 min at 30 °C in a water-saturated atmosphere. Then the dough piece was rolled (roll gap: 0.8 cm), folded in half, rounded for five cycles, and put into an aluminium baking pan for the second dough rest for 40 min under the same conditions as before. Baking took place in a deck oven Type-9110-0082 (Binder, Tuttlingen, Germany) at 230 °C for 18 min.

The breads were either analysed after cooling (2 h) or they were stored at 22 °C under vacuum until further analysis. Amylase activity was determined after a storage time of 30 h. To identify and quantitate sugars in the samples they were either extracted directly after cooling (2 h) or after storage for 22 h, 48 h and 96 h at 22 °C. The baking experiments were carried out with four replicates.

Quantitation of mono-, di-, and oligosaccharides in wheat bread crumb

Mono-, di- and oligosaccharides were extracted from a sample taken from the centre of the bread crumb (0.5 g of dry matter) using 4.0 mL of 50% (v/v) methanol while stirring for 30 min at 22 °C followed by centrifugation at 3500×*g* and 20 °C for 20 min. The solvent was removed using a rotary vacuum concentrator (37 °C, 9 h, 800 Pa). The dry samples were reconstituted in 1.0 mL deionized water and clean-up was performed using strong cation exchange columns (StrataX-C 33 µm, 200 mg, 3 mL, Phenomenex). The columns were activated with methanol (1 × 3.0 mL) and washed with 0.1% (v/v) FA (3 × 3.0 mL). After loading the sample, the sugars were eluted with 0.1% (v/v) FA (2 × 2.0 mL). The eluent was removed using a rotary vacuum concentrator (37 °C, 9 h, 800 Pa) and the sugars were redissolved in deionized water for further analysis.

Mono-, di- and oligosaccharides were analysed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a Dionex ICS-5000 system (Thermo Fisher Scientific). The HPAEC-PAD system was equipped with a CarboPac PA 100 column (250 × 4 mm) connected to a CarboPac PA 100 guard column (50 × 4 mm). Elution was performed with 1.0 mol/L sodium acetate solution (solvent A), 1.0 mol/L sodium hydroxide solution (solvent B) and deionized water (solvent C) at a flow rate of 1.0 mL/min. Solvent B was set to 15% for the total run time. The gradient was 0 min, 0.5% A; 2 min, 0.5% A; 8 min, 10% A; 18 min, 20% A; 23 min, 20% A; 24 min, 0.5% A; 39 min, 0.5% A. PAD detection was carried out with a gold working electrode operating with a standard quadruple waveform. Chromeleon software 7.2 (Thermo Fisher Scientific) was used for system control and data analysis. Quantitation of the sugars was performed by means of external calibration with standard solutions.

Statistical analysis

The sugar contents of the breads containing one amylase preparation each are presented as mean value ± standard deviation. Linear mixed models were used to explore possible differences of the sugar contents between the time points (2 h, 22 h, 48 h and 96 h after baking). The Bonferroni correction was applied to account for multiple testing. Statistical analyses were performed with R statistical software, version 3.6.1. (R Core Team, Vienna, Austria).

Results

Identification of amylases and the protein composition of the preparations

One maltotetraogenic amylase from *P. saccharophila* (UniProtKB accession P22963) was identified in preparations (I) and (II), respectively. In addition, an α-amylase (D9J2M5) was identified in preparation (II). All maltogenic α-amylase preparations (III)–(VII) contained P19531, a maltogenic α-amylase from *G. stearothermophilus* and no additional amylolytic enzyme was detected (Table 2).

The composition of the preparations is shown in Figs. 1 and 2. Comparing the relative iBAQ values for preparation (I), the amylase P22963 accounted for the largest share (87.8%) of all proteins, followed by 7.5% of proteins from *Bacillus licheniformis* and 2.1% of other proteins from *P. saccharophila.* In preparation (II), the major proportion of 71.1% was P22963 followed by other *P. saccharophila* proteins with 12.4%. The α-amylase D9J2M5 from *Aspergillus oryzae* accounted for 1.6% whereas other *Aspergillus oryzae* proteins were present with 4.4%. Preparations (I) and (II) additionally contained other wheat proteins [2.6% for (I) and 10.5% for (II)].

Table 2 Amylases identified in the preparations with their UniprotKB accession number, the number of peptides identified per amylase, the corresponding protein coverage and the organism to which the amylase was assigned

Preparation	Protein	Enzyme	No. of peptides	Coverage (%)	Organism
(I)	P22963	EC 3.2.1.60	25	29.6	*Pelomonas saccharophila*
(II)	P22963	EC 3.2.1.60	16	23.0	*Pelomonas saccharophila*
	D9J2M5	EC 3.2.1.1	16	35.7	*Aspergillus oryzae*
(III)	P19531	EC 3.2.1.133	31	61.5	*Geobacillus stearothermophilus*
(IV)	P19531	EC 3.2.1.133	49	60.8	*Geobacillus stearothermophilus*
(V)	P19531	EC 3.2.1.133	44	53.0	*Geobacillus stearothermophilus*
(VI)	P19531	EC 3.2.1.133	31	49.4	*Geobacillus stearothermophilus*
(VII)	P19531	EC 3.2.1.133	38	60.5	*Geobacillus stearothermophilus*

All protein scores were > 150

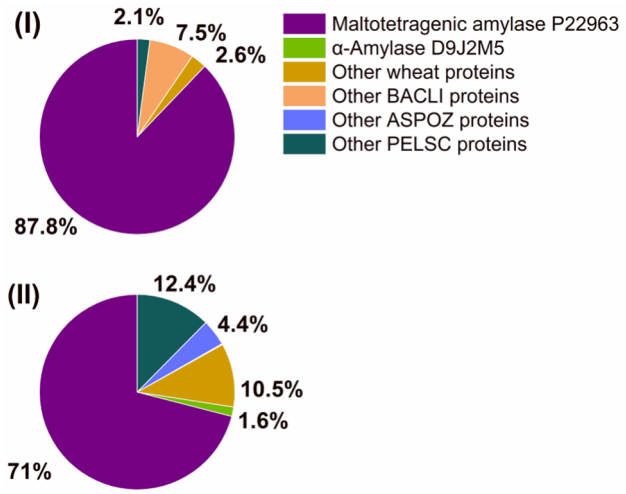

Fig. 1 Relative distribution of wheat and microbial proteins in the maltotetraogenic amylase preparations based on MaxQuant iBAQ values. (I) and (II) are *Pelomonas saccharophila* (PELSC) maltotetraogenic amylase preparations. Other microbial proteins detected are from *Bacillus licheniformis* (BACLI) and *Aspergillus oryzae* (ASPOZ)

All maltogenic α-amylase preparations (Fig. 2) contained P19531 with a share of ≥ 90% [(IV) and (VII)), about 50% ((V) and (VI)] or comparably low with 5.4% (III). Preparations (IV) and (VII) contained exclusively proteins from *G. stearothermophilus*. Only preparations (III) and (VI) contained wheat proteins besides P19531 and other *G. stearothermophilus* proteins. Preparation (III) contained 53.4% gluten and 41.2% other wheat proteins and preparation (VI) 22.8% gluten proteins and 30.8% other wheat proteins. Preparation (V) contained 34.9% proteins from *Bacillus atrophaeus.*

Based on the iBAQ values, the quantity of P22963 from *P. saccharophila* added in the baking experiments was 0.32 mg for preparation (I) and 0.05 mg for (II). Accordingly, the absolute amount of P19531 from the maltogenic α-amylase preparations in the baking recipe ranged from 0.01 mg (III) to 1.21 mg (VII).

Composition of mono-, di-, and oligosaccharides in bread without amylase addition

The composition of mono-, di-, and oligosaccharides in bread crumb without addition of exogenous amylases (control) was already reported by Rebholz et al. [21]. The contents were as follows, sorted in descending order: maltose: 14.6 mg/g_{dm} (± 1.1), sucrose: 5.8 mg/g_{dm} (± 0.8), glucose: 2.9 mg/g_{dm} (± 0.6), fructose: 2.8 mg/g_{dm} (± 2.0), maltotriose: 1.5 mg/g_{dm} (± 0.1) and maltotetraose: 1.1 mg/g_{dm} (± 0.3). No significant changes in the content of the detected sugars were detected during 48 h of storage.

Composition of mono-, di-, and oligosaccharides in breads with amylase addition and changes during storage

The total sugar content was 39.1 mg/g_{dm} (± 3.8) for preparation (I) (Fig. 3) and 58.9 mg/g_{dm} (± 5.4) for preparation (II) 2 h after baking (Fig. 4). The glucose content did not increase significantly from 2 to 22 h, but increased significantly from 22 to 48 h [3.8 mg/g_{dm} (± 0.3) to 4.8 mg/g_{dm} (± 0.4)] in the bread containing preparation (I). The content of maltotriose increased significantly from 22 h [0.2 mg/g_{dm} (± 0.2)] to 48 h [1.6 mg/g_{dm} (± 0.5)], while the content of fructose, maltose and maltotetraose did not show significant changes from 2 to 48 h of storage. Glucose, fructose, sucrose, maltose, maltotriose and maltotetraose were detected 2 h after baking in the bread containing preparation (II). In contrast to preparation (I), maltopentaose, maltohexaose, maltoheptaose and maltooctaose were additionally detected in the bread crumb after 48 h. The maltotetraose content increased significantly from 2.1 mg/g_{dm} (± 1.7) to 29.5 mg/g_{dm} (± 2.0), so that it was the main sugar in the bread manufactured with (II) 96 h after baking.

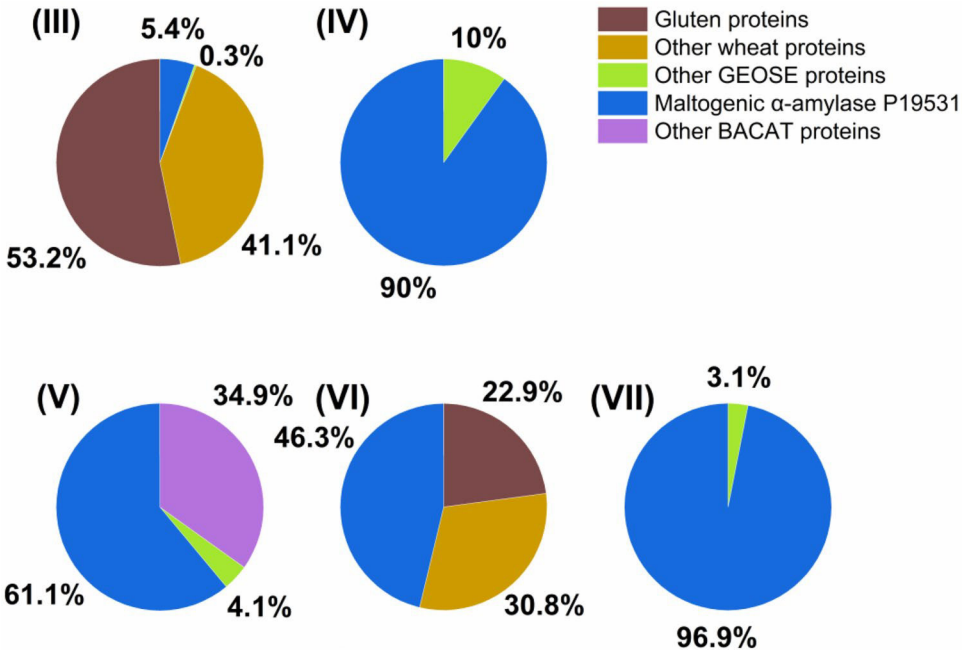

Fig. 2 Relative distribution of wheat and microbial proteins in the maltogenic α-amylase preparations based on MaxQuant iBAQ values. All preparations contain the maltogenic α-amylase from *Geobacillus stearothermophilus* (GEOSE). Other microbial proteins detected are from *Bacillus atrophaeus* (BACAT)

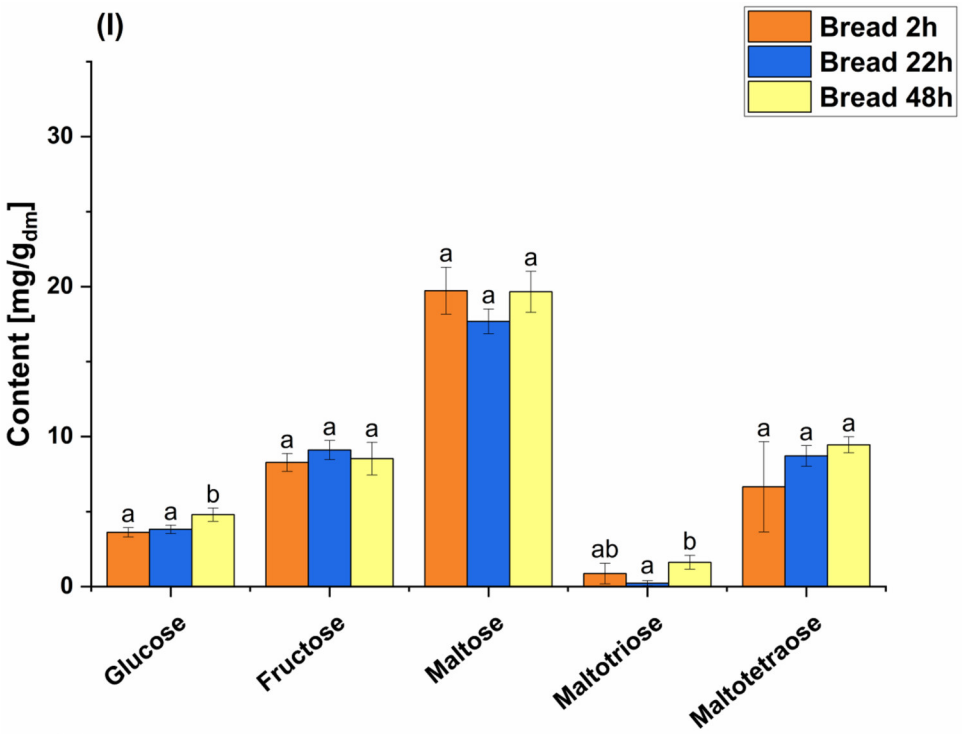

Fig. 3 Sugar content of model breads manufactured with addition of (I), maltotetraogenic amylase preparation from *Pelomonas saccharophila.* Samples were taken 2 h, 22 h, and 48 h after baking. Values are displayed as means ± standard deviation ($n = 4$). Different letters represent significant differences in content for each sugar, respectively ($p < 0.05$)

Glucose, fructose, sucrose, maltose, maltotriose and maltotetraose were identified 2 h after baking in all breads manufactured with exogenous maltogenic α-amylase (Figs. 5, 6), except for breads with (IV) where maltotetraose was identified for the first time at 22 h after baking. Additionally, maltopentaose was detected after 48 h and maltohexaose after 96 h of storage in the samples containing preparation (VI). The total sugar content ranged from 55.3 mg/g$_{dm}$ (±4.4) (V) to 108.4 mg/g$_{dm}$ (±1.6) (VI) after 2 h. Maltose contents ranged from 25.3 mg/g$_{dm}$ (±1.9) for (V) to 50.0 mg/g$_{dm}$ (±4.4) for (VII) 2 h after baking. No significant increase in maltose content was detected between 2 and 96 h in the breads manufactured with (III) and (VII). For preparation (IV), the maltose content increased significantly

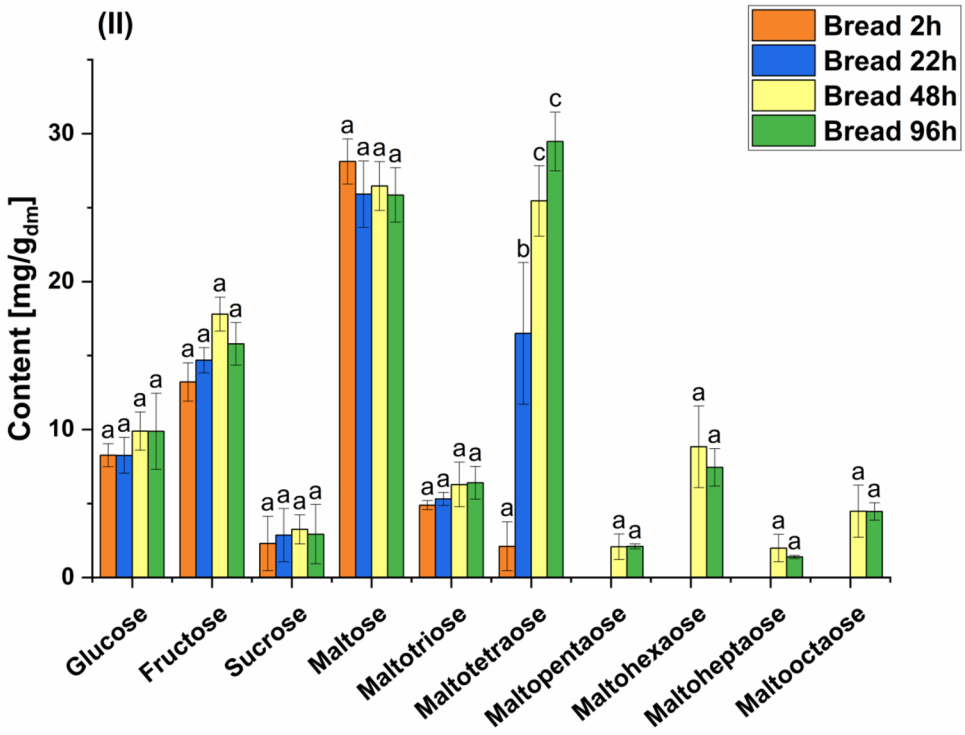

Fig. 4 Sugar content of model breads manufactured with addition of (II), maltotetraogenic amylase preparation from *Pelomonas saccharophila.* Samples were taken 2 h, 22 h, 48 h, and 96 h after baking. Values are displayed as means ± standard deviation ($n = 4$). Different letters represent significant differences in content for each sugar, respectively ($p < 0.05$)

from 27.7 mg/g_{dm} (± 0.8) up to 38.8 mg/g_{dm} (± 8.6), for (V) from 25.3 mg/g_{dm} (± 1.9) to 40.4 mg/g_{dm} (± 0.7) and for (VI) from 37.0 mg/g_{dm} (± 3.2) to 68.6 mg/g_{dm} (± 4.9) considering 2 h and 96 h of storage. The maltotetraose content of the bread crumbs containing preparations (V), (VI) and (VII) increased significantly between 2 and 96 h of storage. The increase was from 5.9 mg/g_{dm} (± 3.9) to 11.9 mg/g_{dm} (± 2.2) for (V), from 9.7 mg/g_{dm} (± 1.7) to 44.1 mg/g_{dm} (± 6.6) for (VI) and from 0.6 mg/g_{dm} (± 0.2) to 22.9 mg/g_{dm} (± 9.3) for (VII). No significant changes in the content of sucrose were observed during storage of the breads containing either of the maltogenic α-amylase preparations.

Residual activity of amylases in model wheat bread

The endogenous activity of the wheat flour used for the baking experiments was 4.1 nkat/g_{dm} (± 0.7) for α-amylase and 378.4 nkat/g_{dm} (± 6.1) for β-amylase. As we have shown before, no cereal amylase activity was detected in the crumb of the control breads after baking [21].

For the maltotetraogenic amylase preparation, (I) we detected 85.8 nkat/g_{dm} (± 11.6) and for preparation (II) 31.7 nkat/g_{dm} (± 6.6). After baking, the residual amylase activity was 0.9 nkat/g_{dm} (± 0.3) in (I) and 0.6 nkat/g_{dm} (± 0.2) in (II). This corresponds to a relative residual activity of 1.1% for (I) and 1.9% for (II) compared to the amylase activity originally used in the dough (Table 1). The activity of maltogenic α-amylase preparations applied to the dough was between 22.0 nkat/g_{dm} (± 0.7) for preparation (III) and 42.9 nkat/g_{dm} (± 2.0). All tested maltogenic α-amylase preparations showed a residual amylase activity (Table 1). Based on the absolute values, the residual activity was between 5.7 nkat/g_{dm} (± 1.2) for (VII) and 14.3 nkat/g_{dm} (± 3.8) for (VI). Compared to the activities applied to the doughs, the relative residual amylase activity in the breads ranged from 13.9% in the breads with (VII) to 45.5% for (VI).

Discussion

In this study, we combined discovery-driven proteomics with the quantitation of mono-, di- and oligosaccharides in bread crumb and the determination of residual amylase activity to get insights into changes in sugar composition caused by the action of exogenous maltotetraogenic amylases and maltogenic α-amylases during straight dough wheat bread making and storage.

For the group of maltotetraogenic amylase and the group of maltogenic α-amylase preparations, one amylase each was identified as being responsible for the amylolytic activity. Our results are in accordance with Prandi et al. who also identified P19531, a maltogenic α-amylase from *G. stearothermophilus,* as being responsible for the amylolytic activity in maltogenic α-amylase preparations applied in baking [28]. To the best of our knowledge, we are the first to analyze maltotetraogenic amylase preparations with proteomics tools and we identified a single amylase P22963 as cause of the amylolytic activity. The iBAQ evaluation was

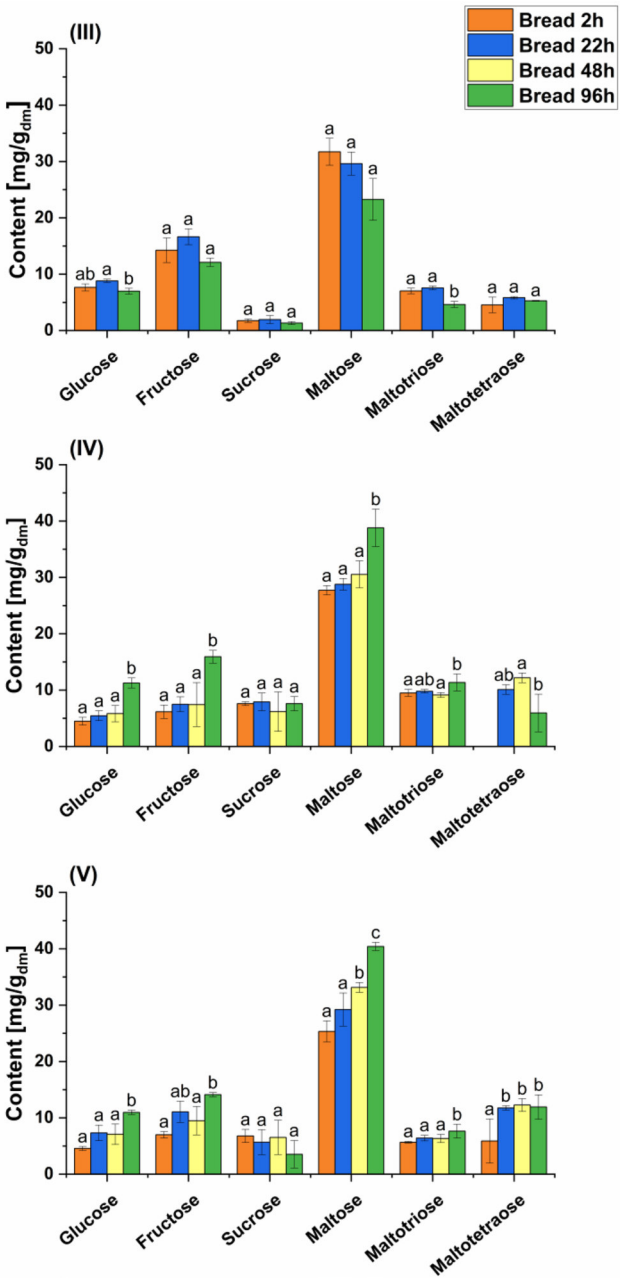

Fig. 5 Sugar content of model breads manufactured with addition of maltogenic α-amylase preparations from *Geobacillus stearothermophilus* (III), (IV) and (V). Samples were taken 2 h, 22 h, 48 h, and 96 h after baking. Values are displayed as means ± standard deviation ($n = 4$). Different letters represent significant differences in content for each sugar, respectively ($p < 0.05$)

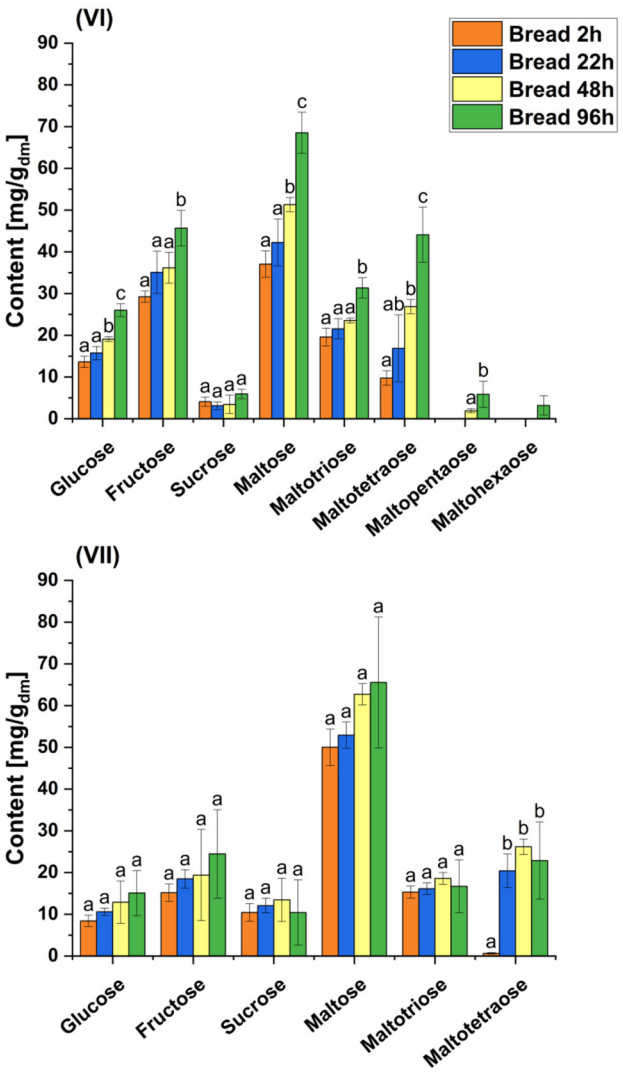

Fig. 6 Sugar content of model breads manufactured with addition of maltogenic α-amylase preparations from *Geobacillus stearothermophilus* (VI) and (VII). Samples were taken 2 h, 22 h, 48 h, and 96 h after baking. Values are displayed as means ± standard deviation ($n = 4$). Different letters represent significant differences in content for each sugar, respectively ($p < 0.05$)

shown to be suitable for the relative quantitation of several different proteins in one sample [26, 29]. The proportion of maltotetraogenic amylase in the preparations (I) and (II) was 87.8% and 71.0%, respectively. Both preparations also contained 4–7% of proteins from other microorganisms and non-gluten proteins, such as the starch synthases P27736 and Q8W2G8, indicating that wheat starch was used as a filler material [30]. The maltogenic α-amylase preparations showed large differences in composition considering that the percentage of maltogenic α-amylase ranged from 5.4% (III) up to 96.9% (VII).

Although both groups of preparations, maltotetraogenic amylase and maltogenic α-amylase, contained the same active enzyme, respectively, the effects on the sugar composition of the bread crumb were quite different. A higher initial activity applied to the dough resulted in a higher maltotetraose content in the bread crumb using the maltotetraogenic amylase preparations (I) and (II). The activity applied to the dough of (I) was 85.8 nkat/g$_{dm}$ and 171% higher than the activity of (II) with 31.7 nkat/g$_{dm}$. Corresponding to that,

the content of the main reaction product, maltotetraose, was about 219% higher with a mean of 6.7 mg/g_{dm} (I) compared to 2.2 mg/g_{dm} (II) 2 h after baking. A similar result was described by Bae et al. when using *Pseudomonas saccharophila* maltotetraogenic amylase. An increase of 200% of maltotetraogenic amylase activity in whole wheat bread making resulted in an increase of 145% of maltotetraose in their study [31].

Overall, only minor changes in the sugar composition were detected during the 48 h of storage of the breads containing preparation (I) and especially no changes in maltotetraose content. This agrees well with the results of Bosmans et al. [8] who also observed no significant changes in the content of maltooligosaccharides in wheat bread during storage using *Pseudomonas saccharophila* maltotetraogenic amylase. Bosmans et al. [8] and Derde et al. [9, 14] assumed inactivation of the maltotetraogenic amylase during baking. We detected a very low residual activity (1.1% of the applied activity) for (I), but apparently this did not cause major changes in the sugar composition of the bread during storage. Opposed to that, the residual activity of (II) was also very low (1.9%), but the absolute maltotetraose content increased from 2.1 mg/g_{dm} 2 h after baking up to 29.5 mg/g_{dm} after 96 h. Thus, maltotetraose increased by 1112% during 48 h and 1300% during 96 h of storage. An additional effect was the formation of maltopentaose, maltohexaose, maltoheptaose and maltooctaose after 48 h and 96 h. These sugars were not present up until 22 h of storage and could be products caused by endohydrolysis of starch chains [9].

Besides its exo-action on starch molecules, *G. stearothermophilus* maltogenic α-amylase performs various endo-attacks on starch [9], with maltose and higher maltooligosaccharides as main products [8, 32]. Our study also confirmed these findings, because the content of maltose and maltooligosaccharides (40.0 mg/g_{dm} for (V) to 66.0 mg/g_{dm} for (VII)) was higher in all breads with maltogenic α-amylase preparations 2 h after baking compared to the control bread (17.3 mg/g_{dm}). In addition, preparation (VI) caused the formation of maltopentaose and maltohexaose, which were not detected in the control. It is also known that maltogenic α-amylase can release glucose from amylose and amylopectin [32]. Therefore, the significant increase of glucose in breads made with the preparations (IV), (V), and (VI) could be a possible consequence of the residual activity.

The main action of maltogenic α-amylase on starch takes place during bread baking, but changes in maltose content during cooling and storage have also been reported for wheat bread [8, 15]. We observed significant changes for maltose and higher maltooligosaccharides for all maltogenic α-amylase preparations during storage (Figs. 5, 6), but these changes were not always the same. There was no direct correlation between the level of residual activity and the amount of sugars formed during storage for the breads made with of maltogenic α-amylase. Using preparation (IV), maltotetraose was identified for the first time at 22 h, whereas(VI) showed the largest impact on the sugar spectrum of wheat bread compared to the control, including the release of maltooligosaccharides after 96 h. In contrast, no significant increase in the sugar concentrations was determined for the breads made with preparation (III), although a residual activity of 42.3% was detected. The absolute contents of maltogenic α-amylase added in the baking experiments ranged from 0.01 mg (III) to 1.21 mg (VII), but there was no direct correlation between the content and the residual activity. This might be due to differences inherent in the preparations we used and could be studied using various dosages of each preparation. However, the intent of our study was not to provide a detailed comparison of the different preparations, but rather show how sugar concentrations are affected when using each preparation at the recommended dosage.

Considering the applied activities in bread dough (Table 1), the relative residual maltogenic α-amylase activities were between 13.9% for (VII) and 45.5% for (VI) and are likely to be responsible for the changes in mono-, di- and oligosaccharide content observed during 96 h of storage. Reichenberger et al. found a comparable relative residual activity of 17.8% for maltogenic α-amylase in their wheat baking experiment [16]. Whether the detected residual activity is related to the characteristics of bread staling or bread crumb properties must be clarified in further studies. We showed that measurements of residual enzymatic activities alone cannot provide sufficient information to evaluate a potential technological function in the final product.

Conclusion

In this study, we identified the specific amylases in two maltotetraogenic amylase and five maltogenic α-amylase preparations commonly applied in wheat bread making. Due to their high temperature stability, a low to medium residual amylolytic activity was found in the bread crumbs containing each of the preparations. Significant changes for glucose, maltose, and higher maltooligosaccharides were observed during storage for up to 96 h and these were most likely caused by the residual amylolytic activity. By applying a methodological approach which targets various amylases and enables the identification of the link between amylases, their residual activity and the resulting changes in the saccharide content of straight dough wheat bread, we provide the foundation for a better understanding of the residual activity of exogenous amylases in wheat bread making.

Acknowledgements The authors thank Stefanie Hackl and Angelika Grassl for excellent technical assistance, Dr. Sabrina Geisslitz (KIT)

for helpful discussions and Dr. Leonie Weinhold (IMBIE, University Hospital Bonn) for advice on statistical evaluation.

Author contributions Conceptualization: GFR and KAS; methodology: GFR and KAS; formal analysis and investigation: GFR, KS and SD; writing—original draft and visualization: GFR; writing—review and editing: KS, SD, CD, TH, and KAS; resources: CD and TH; supervision and project administration: KAS.

Funding This IGF Project of the FEI was supported via AiF within the programme for promoting the Industrial Collective Research (IGF) of the German Ministry of Economic Affairs and Energy (BMWi), based on a resolution of the German Parliament. Allianz Industrie Forschung (Project AiF 19543N). Open access funding enabled and organized by Projekt DEAL.

Declarations

Conflict of interest The authors declare no competing financial interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

Informed consent Not applicable.

References

1. Gray JA, Bemiller JN (2003) Bread staling: molecular basis and control. Compr Rev Food Sci Food Saf. https://doi.org/10.1111/j.1541-4337.2003.tb00011.x
2. Goesaert H, Slade L, Levine H, Delcour JA (2009) Amylases and bread firming—an integrated view. J Cereal Sci. https://doi.org/10.1016/j.jcs.2009.04.010
3. Bosmans GM, Lagrain B, Ooms N, Fierens E, Delcour JA (2013) Biopolymer interactions, water dynamics, and bread crumb firming. J Agric Food Chem. https://doi.org/10.1021/jf4010466
4. Ribotta PD, Le Bail A (2007) Thermo-physical assessment of bread during staling. LWT. https://doi.org/10.1016/j.lwt.2006.03.023
5. Hug-Iten S, Fischer F, Conde-Petit B (2003) Staling of bread: role of amylose and amylopectin and influence of starch-degrading enzymes. Cereal Chem. https://doi.org/10.1094/CCHEM.2003.80.6.654
6. Hug-Iten S, Fischer F, Conde-Petit B (2001) Structural properties of starch in bread and bread model systems: influence of an antistaling alpha-amylase. Cereal Chem. https://doi.org/10.1094/CCHEM.2001.78.4.421
7. Martin ML, Hoseney RC (1991) A Mechanism of bread firming. II. Role of starch hydrolyzing enzymes. Cereal Chem 68:503–507
8. Bosmans GM, Lagrain B, Fierens E, Delcour JA (2013) Impact of amylases on biopolymer dynamics during storage of straight-dough wheat bread. J Agric Food Chem. https://doi.org/10.1021/jf402021g
9. Derde LJ, Gomand SV, Courtin CM, Delcour JA (2012) Hydrolysis of β-limit dextrins by α-amylases from porcine pancreas, *Bacillus subtilis*, *Pseudomonas saccharophila* and *Bacillus stearothermophilus*. Food Hydrocolloids. https://doi.org/10.1016/j.foodhyd.2011.06.001
10. Goesaert H, Bijttebier A, Delcour JA (2010) Hydrolysis of amylopectin by amylolytic enzymes: level of inner chain attack as an important analytical differentiation criterion. Carbohydr Res. https://doi.org/10.1016/j.carres.2009.11.011
11. Goesaert H, Brijs K, Veraverbeke WS, Courtin CM, Gebruers K, Delcour JA (2005) Wheat flour constituents. How they impact bread quality, and how to impact their functionality. Trends Food Sci Technol. https://doi.org/10.1016/j.tifs.2004.02.011
12. Amigo JM, Del Olmo AA, Engelsen MM, Lundkvist H, Engelsen SB (2016) Staling of white wheat bread crumb and effect of maltogenic α-amylases. Part 1. Spatial distribution and kinetic modeling of hardness and resilience. Food Chem. https://doi.org/10.1016/j.foodchem.2016.02.162
13. Goesaert H, Leman P, Bijttebier A, Delcour JA (2009) Antifirming effects of starch degrading enzymes in bread crumb. J Agric Food Chem. https://doi.org/10.1021/jf803058v
14. Derde LJ, Gomand SV, Courtin CM, Delcour JA (2012) Characterisation of three starch degrading enzymes: thermostable β-amylase, maltotetraogenic and maltogenic α-amylases. Food Chem. https://doi.org/10.1016/j.foodchem.2012.05.031
15. Lagrain B, Leman P, Goesaert H, Delcour JA (2008) Impact of thermostable amylases during bread making on wheat bread crumb structure and texture. Food Res Int. https://doi.org/10.1016/j.foodres.2008.07.006
16. Reichenberger K, Luz A, Seitl I, Fischer L (2020) Determination of the direct activity of the maltogenic amylase from *Geobacillus stearothermophilus* in white bread. Food Anal Methods. https://doi.org/10.1007/s12161-019-01673-7
17. European Parliament, European Council (2008) Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on food enzymes and amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/9
18. European Parliament, European Council Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004
19. Cornaggia C, Evans DE, Draga A, Mangan D, McCleary BV (2019) Prediction of potential malt extract and beer filterability using conventional and novel malt assays. J Inst Brew. https://doi.org/10.1002/jib.567
20. Uhr L, Buchholz T, Homann T, Huschek G, Rawel HM (2014) Targeted proteomics-based analysis of technical enzymes from fungal origin in baked products. J Cereal Sci. https://doi.org/10.1016/j.jcs.2014.04.007
21. Rebholz GF, Sebald K, Dirndorfer S, Dawid C, Hofmann T, Scherf KA (2021) Impact of exogenous α-amylases on sugar

release on straight dough wheat bread. Eur Food Res Technol. https://doi.org/10.1007/s00217-020-03657-y
22. Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized ppb-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol. https://doi.org/10.1038/nbt.1511
23. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M (2011) Andromeda: a peptide search engine integrated into the MaxQuant environment. J Proteome Res. https://doi.org/10.1021/pr101065j
24. European Food Safety Authority, Parma, IT. https://www.efsa.europa.eu/de/search/site/amylase. Accessed 1 July 2019
25. Food and Drug Administration, Silver Spring, USA. https://www.search.usa.gov/search?query=amylase&affiliate=fda1. Accessed 1 July 2019
26. Schwanhäusser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, Chen W, Selbach M (2011) Global quantification of mammalian gene expression control. Nature. https://doi.org/10.1038/nature10098
27. Kieffer R, Wieser H, Henderson MH, Graveland A (1998) Correlations of the breadmaking performance of wheat flour with rheological measurements on a micro-scale. J Cereal Sci. https://doi.org/10.1006/jcrs.1997.0136
28. Prandi B, Lambertini F, Varani M, Faccini A, Suman M, Leporati A, Tedeschi T, Sforza S (2020) Assessment of enzymatic improvers in flours using LC-MS/MS detection of marker tryptic peptides. J Am Soc Mass Spectrom. https://doi.org/10.1021/jasms.9b00109
29. Geisslitz S, Ludwig C, Scherf KA, Koehler P (2018) Targeted LC–MS/MS reveals similar contents of α-amylase/trypsin-inhibitors as putative triggers of nonceliac gluten sensitivity in all wheat species except einkorn. J Agric Food Chem. https://doi.org/10.1021/acs.jafc.8b04411
30. Ainsworth C, Clark J, Balsdon J (1993) Expression, organisation and structure of the genes encoding the waxy protein (granule-bound starch synthase) in wheat. Plant Mol Biol. https://doi.org/10.1007/BF00038996
31. Bae W, Lee SH, Yoo S-H, Lee S (2014) Utilization of a maltotetraose-producing amylase as a whole wheat bread improver: dough rheology and baking performance. J Food Sci. https://doi.org/10.1111/1750-3841.12538
32. Christophersen C, Otzen D, Normann BE, Christiensen S, Schäfer T (1998) Enzymatic characterisation of Novamyl®, a thermostable α-amylase. Starke. https://doi.org/10.1002/(SICI)1521-379X(199801)50:1%3C39::AID-STAR39%3E3.0.CO;2-S

4. General discussion

4.1 Protein composition of the amylase preparations

The protein composition of the amylase preparations applied was analyzed using iBAQ, a tool implemented in MaxQuant. The values generated by iBAQ are the MS peptide intensities divided by the number of theoretical peptides. Thus, iBAQ values are proportional to the molar quantities of the proteins [132]. It was shown before, that the iBAQ approach is suitable for quantitating proteins from the albumin/globulin fraction in cereal samples and yields comparable results to a targeted analysis of these proteins [133]. There are several targeted [134, 135] and discovery-driven methods [136, 137] described with the aim of quantifying or identifying enzymes in preparations. In contrast to these studies, our aim was to get deeper insights into the amylase preparations and generate knowledge about their protein composition. With that aim, the discovery-driven DDA experiment followed by MaxQuant data evaluation was the method of choice. For each individual amylase preparation, we identified the amylases present, assigned the amylolytic activity to a specific amylase and got information about the origin and the share of other proteins present in the samples.

In the first step of the proteomics data processing, the amylases present in the preparations were identified. In the second step, their share was determined via label-free quantitation. Table 3 lists the identified enzymes in the α-amylase preparations and their organisms of origin. Additionally, the parameters of identification, the number of peptides detected for each protein and the corresponding protein coverage are listed.

Table 3: Identified amylases in the α-amylase preparations with their UniprotKB accession number, the number of peptides identified per amylase, the corresponding protein coverage and the organism to which the amylases were assigned.

Preparation	UniprotKB accession	Enzyme	No. of Peptides	Coverage [%]	Organism
(A)	P00692	α-amylase	33	72.6	*Bacillus amyloliquefaciens*
(B)	A0A0N0H9C2	α-amylase	25	53.1	*Thermoactinomyces vulgaris*
(C)	D9J2M5	α-amylase	27	41.4	*Aspergillus oryzae*
	A0A1S9DG38	glucoamylase	13	21.7	*Aspergillus oryzae*
(D)	D9J2M5	α-amylase	26	34.9	*Aspergillus oryzae*
	A0A1S9DG38	glucoamylase	12	21.6	*Aspergillus oryzae*
(E)	D9J2M5	α-amylase	29	47.6	*Aspergillus oryzae*
	A0A1S9DG38	glucoamylase	11	21.9	*Aspergillus oryzae*

One α-amylase was identified for each of the bacterial α-amylases; in contrast, the fungal α-amylase preparation contained one glucoamylase (A0A1S9DG38) and one α-amylase

(D9J2M5) each. A fungal α-amylase with high similarity to the fungal α-amylase we identified was previously identified by Uhr et al. [134] and Prandi et al. [137].

One amylase was identified in each of the maltotetraogenic amylase preparations and the maltogenic α-amylase preparations: the maltotetraogenic amylase P22963 from *Pelomonas saccharophila* and the maltogenic α-amylase P19531 from *Geobacillus stearothermophilus.* Table 4 lists the identified enzymes in the maltogenic α-amylase and maltotetraogenic amylase preparations in together with the organisms of origin and the parameters of identification, the number of peptides detected for each protein and the corresponding protein coverage.

Table 4: Amylases detected in the maltotetraogenic α-amylase and maltogenic amylase preparations with UniprotKB accession number, the number of peptides detected for each amylase with the corresponding protein coverage, and the organism of origin.

Preparation	UniprotKB accession	Enzyme	No. of Peptides	Coverage [%]	Organism
(I)	P22963	maltotetraogenic amylase	25	29.6	*Pelomonas saccharophila*
(II)	P22963	maltotetraogenic amylase	16	23.0	*Pelomonas saccharophila*
	D9J2M5	α-amylase	16	35.7	*Aspergillus oryzae*
(III)	P19531	maltogenic α-amylase	31	61.5	*Geobacillus stearothermophilus*
(IV)	P19531	maltogenic α-amylase	49	60.8	*Geobacillus stearothermophilus*
(V)	P19531	maltogenic α-amylase	44	53.0	*Geobacillus stearothermophilus*
(VI)	P19531	maltogenic α-amylase	31	49.4	*Geobacillus stearothermophilus*
(VII)	P19531	maltogenic α-amylase	38	60.5	*Geobacillus stearothermophilus*

Prandi et al. also describe P19531 from *Geobacillus stearothermophilus* as the amylolytic enzyme in a maltogenic α-amylase preparation [137]. Thus, the findings within the scope of this work are in line with literature. In case of the maltotetraogenic amylase preparation, to the best of our knowledge, we were the first to describe P22963 from *Pelomonas saccharophila* as the amylase responsible for the activity of a preparation used in wheat bread making.

The α-amylase, maltotetraogenic amylase and maltogenic α-amylase preparations analyzed in this work can be categorized into three groups. The first group are the preparations containing wheat proteins and proteins from the organism the amylase was assigned to. The second group contains preparations with wheat proteins, proteins from the amylase organism and proteins from different microorganisms. The third group contains preparations with exclusively microbial proteins. All bacterial and fungal α-amylase preparations as well as the maltogenic α-amylase preparations (III) and (V) are part of the first group. The maltotetraogenic

preparations represent the second group and the maltogenic α-amylase preparations (IV), (V) and (VII) are in group three.

Focusing on the wheat proteins in the samples it is noticeable, that no gluten proteins were detected in the preparations (A), (I) and (II). While the other preparations of group one contained wheat flour as filler material (based on the gluten and other wheat proteins present), a higher level of the protein P27736, a starch synthase present in wheat starch [138], indicated that (A), (I) and (II) contained wheat starch as filler material. The relative amount of the proteins assigned to the filler materials wheat flour and wheat starch does not give information about their amount in the powdery sample used in this work. Since the extraction method performed was water-based without organic solvents, it targets on easily extractable proteins like enzymes, and not protein polymers like gluten proteins [4]. Thus, the extractability of polymeric proteins was incomplete with the chosen method and was not intended to determine the amount of wheat flour or wheat starch in the sample. However, the extraction is suitable to determine the amount of amylase present and to identify other proteins in the extract due to the targeted extraction of amylases.

Based on the relative amounts of the identified amylases it was possible to assign the activity of the preparation to one amylase, in case of several amylases detected. The amount of the glucoamylase A0A1S9DG38 detected in the fungal α-amylase preparations was < 0.3% and can be neglected compared to the present α-amylase with a share of 53.7% to 79.4%. The same observation was made for the maltotetraogenic amylase preparation (II) where the fungal α-amylase D9J2M5 had a share of 1.6% and the major amylase P22963 from *Pelomonas saccharophila* had a share of 71.0%.

These findings enable the assignment of the amylolytic activity to a specific amylase and provide information about the amylase composition of the studied preparations as well as an overview of further proteins present in the samples.

4.2 Residual activity of amylases in wheat bread crumb

Residual activity in the model wheat breads was measured for (A) *Bacillus amyloliquefaciens* α-amylase, (I) and (II) maltotetraogenic amylase from *Pelomonas saccharophila* and for maltogenic α-amylase from *Geobacillus stearothermophilus* ((III) – (VII)). Regarding the fungal α-amylase preparations no residual activity was measured in the bread crumb. This finding is consistent with the temperature stability of fungal α-amylases being described as low [3]. A high temperature stability has been reported previously for the *Bacillus amyloliquefaciens* α-amylase [139], thus a moderate residual activity was expected. However, we found a very low activity of *Bacillus amyloliquefaciens* in bread crumb with 0.07 nkat/g_{dm} and this corresponds to 4.01% of the applied activity of 1.77 nkat/ g_{dm}.

It is known that amylase from *Pelomonas saccharophila* has a high temperature stability up to 90°C. However, only data on the stability of these maltotetraogenic amylases in starch solutions has been published [57]. So far, no data about their stability in the complex matrix and foamy structure of wheat bread are available. With the formulation and manufacturing parameters we selected in the baking experiments a residual activity was measurable. Further research is needed to confirm these results for large scale baking trials.

The temperature stability of maltogenic α-amylase from *Geobacillus stearothermophilus* in wheat bread has been well studied and the amylase residual activity in the bread crumb has been confirmed in multiple baking trials [57, 93, 95, 103]. In line with these findings, we were also able to demonstrate the residual activity of maltogenic α-amylase from *Geobacillus stearothermophilus* in the baking tests we conducted with the five different preparations in our study.

The residual activity measured for the maltotetraogenic amylases was quite similar, despite the different amounts of dosage of the preparations (0.9 nkat/g_{dm} for (I) and 0.6 nkat/g_{dm} for (II)). This corresponded to a relative residual activity of 1.1% and 1.9%, respectively.

The absolute residual activity of the maltogenic α-amylase preparations ranged from 5.7 nkat/g_{dm} for (VII) to 14.3 nkat/g_{dm} for (VI) with a relative residual activity of 13.9% to 45.5%. Based on the amylase content measured by iBAQ it was possible to determine the amount of amylase (in mg) included in the preparation dosed in the baking experiment. Equal amounts of preparations (III) - (VI), 5.0 mg, were used in the baking tests, and 2.5 mg of preparation (VII) was used. Due to a varying protein content of the preparations, the amylase content within the dosage was quite different. Table 5 gives an overview of the dosages, the amylase contents and the residual activities measured in the bread crumbs. The dosage of amylase preparations in the study was performed according to manufacturers' recommendations and does not represent the actual content of amylases added in the baking experiments. Specifically, it becomes apparent that a dosage of 5 mg results in an actual amylase content between 0.01 mg and 0.04 mg for the maltogenic α-amylase preparations. Therefore, it is not the actual amount of amylase, which is a major parameter for the activity of the amylase, but the actual applied activity. However, a high dosage of activity does not necessarily lead to a higher residual activity, e.g., in the group of maltogenic α-amylase preparations (Table 5), preparation (IV) had the highest dosed activity (42.9 nkat/g_{dm}) with a residual activity of 11.0 nkat/g_{dm}. This residual activity lower than the residual activity of preparation (VI) (14.3 nkat/g_{dm}) whereas the applied activity of (VI) was about 11.4 nkat lower (31.5 nkat) than that of preparation (IV).

Table 5: Protein dosage calculated via the amylase proportions determined by iBAQ as well as the applied activity per g bread and the resulting residual activity for seven preparations. Values for applied amylase activity and residual activity are shown as mean ± standard deviation for n=3 samples.

Prep.	Dosage [mg]	Protein dosage [mg]	Amylase iBAQ [%]	Amylase [mg]	Applied activity [nkat/g_{dm}]	Residual activity [nkat/g_{dm}]	Residual activity [%]
(I)	42.5	0.36	87.8	0.32	85.8 ±11.6	0.9 ±0.3	2.0
(II)	15.0	0.07	71.0	0.05	31.7 ± 6.6	0.6 ±0.2	1.1
(III)	5.0	0.10	5.4	0.01	22.0 ±0.7	9.3 ±1.4	42.3
(IV)	5.0	0.01	90.0	0.01	42.9 ±2.0	11.0 ±5.3	25.7
(V)	5.0	0.03	61.1	0.02	38.6 ±1.7	11.0 ±4.9	28.6
(VI)	5.0	0.09	46.3	0.04	31.5 ±3.2	14.3 ±3.8	45.5
(VII)	2.5	0.01	96.9	0.01	41.0 ±3.8	5.7 ±1.2	13.9

Prep.: Preparation

In our work we did not systematically check the relationships between dosed activity during dough preparation and residual activity in the bread crumb. We showed that a general comparison of different amylase preparations containing different matrices of filler material and different amounts of amylase is not possible. Moreover, it is necessary to focus on each single preparation to determine whether a protein leads to a residual activity in bread. Additionally, within our sample set of preparations, we were able to identify the preparations that showed residual activity in the model breads and we could link this residual activity to the amylase identified in the previous step of the work.

4.3 Sugar release in white bread crumb during storage

Since several amylolytic hydrolysis products are substrates for bakers' yeast, we did not use yeast as a leavening agent. Glucose, maltose and maltotriose can be substrates for yeast, thus a fermentation process would have an impact on the sugar content in the breads. It would not be possible to identify the specific amylolytic effect of the amylase applied if yeast had been added to the dough during bread making. Additionally, yeast comes with a microbiome including enzymes with hydrolytic activity, e.g., the maltase MALS [73], which would have an additional impact on the sugar spectrum in bread. Yeast and its enzymes are inactivated at temperatures above 50°C during baking, thus they have no influence on the sugar formation during baking.

The foamy structure is a key element for a model matrix, comparable to common wheat bread. Therefore, unleavened bread was not taken into consideration and the model breads were leavened by adding baking soda. Due to the carbonate salts baking soda increases the pH of wheat bread. The pH of straight dough wheat bread leavened by yeast was shown to be at

about pH 6 [140]. Wheat doughs made with baking soda were shown to have a pH > 8 [141]. To provide an environment where the pH-sensitive amylases have a similar rate of metabolism compared to yeast-leavened bread, the pH of the model breads was adjusted by a buffer solution with 0.1 mol/L citric acid monohydrate and 0.1 mol/L trisodium citrate dihydrate at pH 2.75.

Regarding the sugar content of the control breads (breads without any amylase addition) no changes in the sugar spectrum during storage were detected. The sugars present were glucose, fructose, sucrose, maltose, maltotriose and maltotetraose with maltose as the predominant sugar considering its content.

For all preparations analyzed within this work, maltose was shown to be the predominant sugar after baking. The amount of maltose in the breads made with exogenous α-amylase was between 20.27 mg/g_{dm} and 70.93 mg/g_{dm}. For maltogenic α-amylase and maltotetraogenic amylase the content ranged from 17.73 mg/g_{dm}. to 50.03 mg/g_{dm}. Exogenous α-amylase was shown to have a increasing effect on maltose levels in wheat bread baking [74]. The increasing effect on maltose content resulting from maltogenic α-amylase application was due to its main hydrolysis product maltose. For maltotetraogenic amylase it was shown, that the enzyme releases maltose as well as maltotetraose from wheat starch [57].

Despite the lack of residual activity, changes in the sugar spectrum were measurable for the fungal α-amylase preparations and the α-amylase from *Thermoactinomyces vulgaris*. These changes were significant increases in glucose, fructose and maltose for (B), maltotetraose for (C), glucose, fructose, maltose and maltotriose for (D) and (E). An amylolytic residual activity cannot be responsible for these changes, thus it is assumed that retrogradation of starch might be involved in the extractability of mono-, di-, and oligosaccharides. This is in line with the findings of Lin and Lineback [142], who described a changing amount of extractable maltooligosaccharides in wheat bread during a storage time of six days. Plasticizers such as sugars have an impact on the denaturation and melting behavior of biopolymers like starch. The kinetics of this mechanism are related to the effective molar density of hydroxyl groups available for intermolecular hydrogen bonding [143]. Thus, sugars can have an influence on the volumetric density of intermolecular hydrogen bonds via their properties as plasticizers involved in gelatinization [144]. This can result in a weakening of hydrogen bonds during retrogradation of wheat starch which can be responsible for the changes in extractability of mono-, di-, and oligosaccharides from wheat bread crumb during storage. This effect occurs within all analyzed breads including the control bread. However, the described effect is expected to be smaller for the control bread, due to a considerably lower overall sugar content compared to the breads made with amylase.

Nevertheless, differences regarding the sugar release from wheat breads supplemented with exogenous α-amylase were identified. In contrast to the breads without residual activity in the crumb, the breads with *Bacillus amyloliquefaciens* α-amylase showed major changes after 48 h of storage. The following five maltooligosaccharides, not detected before, were present from the time point 48 h on: maltotetraose, maltopentaose, maltohexaose, maltoheptaose and maltooctaose which are all typical hydrolysis products of α-amylase [145]. Considering that the *Bacillus amyloliquefaciens* α-amylase preparation was the only one within the sample set of α-amylase preparations showing a residual activity and release of sugars not detected before it is strongly presumed that it can be traced back to amylase residual activity.

For all preparations within the group of maltotetraogenic amylase and maltogenic α-amylase preparations a residual activity was measured in the model breads. The observed changes within the groups with regard to the composition of the sugar spectrum and the level of the sugar contents were very different.

The residual activity of the maltotetraogenic amylase preparations (I) and (II), which was 1.1% and 1.9% of the applied activity, respectively, resulted in a varying impact on the sugar spectrum. For (I) no major changes in sugar composition were detected, for (II) the maltotetraose content increased by 1300% during 96 h of storage and a formation of maltopentaose, maltohexaose, maltoheptaose and maltooctaose after 48 h and 96 h was determined. Thus, in case of the maltotetraogenic preparations the same protein, with residual activities in a comparable range, resulted in a different effect on the sugar content. All sugars formed during the storage of (II) are likely to be hydrolysis products of maltotetraogenic amylase [55].

Regarding the maltogenic α-amylase preparations, significant changes of maltose and maltooligosaccharides during storage were observed, but no uniform pattern of change was evident. Considering the overall amount of maltooligosaccharides present in the breads 2 h after baking, the preparations can be categorized into two groups, whereby preparations (III), (IV) and (V) as well as (VI) and (VII) can be grouped. For the first group the amount of maltooligosaccharides after baking was within a range of 36.97 mg/g_{dm} (V) and 44.02 mg/g_{dm} (III). In group two, with 61.47 mg/g_{dm} for preparation (VI) and 66.00 mg/g_{dm} for preparation (VII) the amounts of these sugars were substantially higher than for preparations of the first group. Additionally, no significant increases in the sugar concentrations were determined for (III). Preparations (IV) and (V) showed significant changes in the sugars glucose, fructose, maltose, maltotriose and maltotetraose. (VI) not only had the highest initial sugar content, it also showed major changes regarding the sugar composition including the release of maltooligosaccharides after 96 h. Table 6 shows the applied activities, the resulting residual activities of maltogenic α-amylase preparations containing P19531 from *Geobacillus stearothermophilus* as well as

the amount of maltooligosaccharides after baking and the maltooligosaccharides affected by changes during storage.

Table 6: Overview of applied activities, residual activities of maltogenic α-amylase preparations and their effect on the sugar spectrum of model breads. Values are displayed as means ± standard deviation (n = 3).

Prep.	Applied activity [nkat/g_{dm}]	Residual activity [nkat/g_{dm}]	Residual activity [%]	Maltooligosaccharides at 2 h [mg/g_{dm}]	Major storage changes at 96 h:
(III)	22.0 ±0.7	9.3 ±1.4	42.33	44.02 ± 3.37	Maltotriose
(IV)	42.9 ±2.0	11.0 ±5.3	25.70	37.20 ± 1.33	Maltose, maltotriose, maltotetraose
(V)	38.6 ±1.7	11.0 ±4.9	28.63	36.97 ± 4.10	Maltose, maltotriose, maltotetraose
(VI)	31.5 ±3.2	14.3 ±3.8	45.45	61.47 ± 1.43	Maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose
(VII)	41.0 ±3.8	5.7 ±1.2	13.94	65.99 ± 5.77	Maltotetraose

Prep.: Preparation

By comparing preparations (IV) and (VII) it can be shown, that the activity did not generally determine the amount of maltooligosaccharides formed during baking. Complementary to that, it is not possible to draw a general conclusion from the residual activity to a certain pattern of change during the aging process. For example, preparation (III) had a similar absolute residual activity as (VII), but a different sugar was affected by the changes. It can be assumed that an increasing residual activity also leads to increased changes during storage. To prove this association, further experiments using the same preparations are needed.

The plasticizing impact of sugars on intermolecular hydrogen bonds and thus on the extractability of sugars from bread described above is present for both the maltotetraogenic amylase samples and the maltogenic α-amylase samples. However, it is assumed that the effects resulting from a residual amylase activity on the sugar spectrum exceed the impact of extractability.

Some limitations of this work are outlined in the following. First, we applied preparations containing the same amylase, but we did not apply different dosages of the same preparation itself. Thus, the preparations differed in the ingredients and the date of manufacturing. It was shown that the condition and conformation of the starch has a major influence on the activity of the amylases during baking [146]. Second, the bread itself with its pore size and thermal conductivity affects the inactivation degree of amylases and thus the kinetics of residual activity

[97]. Although the baking trials were standardized, it is not possible to guarantee the exact same conditions, which may be another explanation for differences in sugar release caused by the same type of amylase preparations.

Nevertheless, it was shown within this work that significant changes for glucose, maltose and higher maltooligosaccharides can be observed during storage of wheat bread for up to 96 h and that these changes can be linked to residual amylolytic activity.

5. References

1. Dupont FM, Altenbach SB (2003) Molecular and biochemical impacts of environmental factors on wheat grain development and protein synthesis. *J. Cereal Sci.* https://doi.org/10.1016/S0733-5210(03)00030-4
2. Tebben L, Shen Y, Li Y (2018) Improvers and functional ingredients in whole wheat bread. A review of their effects on dough properties and bread quality. *Trends Food Sci Technol.* https://doi.org/10.1016/j.tifs.2018.08.015
3. Whitehurst RJ, van Oort M (eds) (2010) Enzymes in Food Technology. *Wiley-Blackwell*, Chichester
4. Goesaert H, Brijs K, Veraverbeke WS, Courtin CM, Gebruers K, Delcour JA (2005) Wheat flour constituents. How they impact bread quality, and how to impact their functionality. *Trends Food Sci Technol.* https://doi.org/10.1016/j.tifs.2004.02.011
5. Henrissat B (1991) A classification of glycosylhydrolases based on aminoacid sequence similarities. *Biomed. J.* https://doi.org/10.1042/bj2800309
6. Törrönen A, Rouvinen J (1997) Structural and functional properties of low molecular weight endo-1,4-beta-xylanases. *J. Biotechnol.* https://doi.org/10.1016/s0168-1656(97)00095-3
7. Courtin CM, Delcour JA (2002) Arabinoxylans and endoxylanases in wheat flour bread-making. *J. Cereal Sci.* https://doi.org/10.1006/jcrs.2001.0433
8. Simpson DJ, Fincher GB, Huang AH, Cameron-Mills V (2003) Structure and Function of Cereal and Related Higher Plant (1,4)-β-Xylan Endohydrolases. *J. Cereal Sci.* https://doi.org/10.1006/jcrs.2002.0488
9. Cleemput G, Bleukx W, van Oort M, Hessing M, Delcour J (1995) Evidence for the presence of arabinoxylan hydrolysing enzymes in European wheat flours. *J. Cereal Sci.* https://doi.org/10.1016/0733-5210(95)90043-8
10. Cauvain S (2015) Technology of Breadmaking. *Springer International Publishing,* Cham
11. Courtin CM, Gelders G.G., Delcour JA (2001) Use of Two Endoxylanases with Different Substrate Selectivity for Understanding Arabinoxylan Functionality in Wheat Flour Breadmaking. *Cereal Chem*. https://doi.org/10.1094/CCHEM.2001.78.5.564
12. Courtin CM, Delcour JA (2001) Relative Activity of Endoxylanases Towards Water-extractable and Water-unextractable Arabinoxylan. *J. Cereal Sci.* https://doi.org/10.1006/jcrs.2000.0354

13. Rouau X, El-Hayek ML, Moreau D (1994) Effect on an Enzyme Preparation Containig Pentosanases on the Bread-making Quality of Flours in Relation to Changes in Pentosan Properties. *J. Cereal Sci.* https://doi.org/10.1006/jcrs.1994.1033

14. Casas-Godoy L, Gasteazoro F, Duquesne S, Bordes F, Marty A, Sandoval G (2018) Lipases: An Overview. *Methods Mol. Biol*. https://doi.org/10.1007/978-1-61779-600-5_1

15. Gerits LR, Pareyt B, Decamps K, Delcour JA (2014) Lipases and Their Functionality in the Production of Wheat-Based Food Systems. *Compr. Rev. Food Sci. Food Saf.* https://doi.org/10.1111/1541-4337.12085

16. Gerits LR, Pareyt B, Masure HG, Delcour JA (2015) Native and enzymatically modified wheat (Triticum aestivum L.) endogenous lipids in bread making. A focus on gas cell stabilization mechanisms. *Food Chem*. https://doi.org/10.1016/j.foodchem.2014.09.064

17. Gerits LR, Pareyt B, Delcour JA (2013) Single run HPLC separation coupled to evaporative light scattering detection unravels wheat flour endogenous lipid redistribution during bread dough making. *LWT.* https://doi.org/10.1016/j.lwt.2013.03.015

18. Pomeranz Y, Chung OK (1978) Interaction of lipids with proteins and carbohydrates in breadmaking. *J Amer Oil Chem Soc.* https://doi.org/10.1007/BF02676944

19. McCann TH, Small DM, Batey IL, Wrigley CW, Day L (2009) Protein–lipid interactions in gluten elucidated using acetic acid fractionation. *Food Chem.* https://doi.org/10.1016/j.foodchem.2008.11.070

20. Koehler P (2001) Study of the Effect of DATEM. 3: Synthesis and Characterization of DATEM Components. *LWT*. https://doi.org/10.1006/fstl.2000.0690

21. Melis S, Meza Morales WR, Delcour JA (2019) Lipases in wheat flour bread making: Importance of an appropriate balance between wheat endogenous lipids and their enzymatically released hydrolysis products. *Food Chem*. https://doi.org/10.1016/j.foodchem.2019.125002

22. Gerits LR, Pareyt B, Delcour JA (2014) A lipase based approach for studying the role of wheat lipids in bread making. *Food Chem.* https://doi.org/10.1016/j.foodchem.2014.01.107

23. Schaffarczyk M, Østdal H, Koehler P (2014) Lipases in wheat breadmaking. Analysis and functional effects of lipid reaction products. *J. Agric. Food Chem.* https://doi.org/10.1021/jf5022434

24. Bankar SB, Bule MV, Singhal RS, Ananthanarayan L (2009) Glucose oxidase--an overview. *Biotechnol. Adv.* https://doi.org/10.1016/j.biotechadv.2009.04.003

25. Joye IJ, Lagrain B, Delcour JA (2009) Use of chemical redox agents and exogenous enzymes to modify the protein network during breadmaking – A review. *J. Cereal Sci.* https://doi.org/10.1016/j.jcs.2009.04.001

26. Hanft F, Koehler P (2006) Studies on the effect of glucose oxidase in bread making. *J. Sci. Food Agric.* https://doi.org/10.1002/jsfa.2455

27. Dunnewind B, van Vliet T, Orsel R (2002) Effect of Oxidative Enzymes on Bulk Rheological Properties of Wheat Flour Doughs. *J. Cereal Sci.* https://doi.org/10.1006/jcrs.2002.0471

28. Meerts M, van Ammel H, Meeus Y, van Engeland S, Cardinaels R, Oosterlinck F, Courtin CM, Moldenaers P (2017) Enhancing the Rheological Performance of Wheat Flour Dough with Glucose Oxidase, Transglutaminase or Supplementary Gluten. *Food Bioproc Tech*. https://doi.org/10.1007/s11947-017-1986-0

29. Decamps K, Joye IJ, Rakotozafy L, Nicolas J, Courtin CM, Delcour JA (2013) The bread dough stability improving effect of pyranose oxidase from trametes multicolor and glucose oxidase from Aspergillus niger: unraveling the molecular mechanism. *J. Agric. Food Chem.* https://doi.org/10.1021/jf4021416

30. Vemulapalli V, Miller KA, Hoseney RC (1998) Glucose oxidase in breadmaking systems. *Cereal Chem*. https://doi.org/10.1094/CCHEM.1998.75.4.439

31. Fraatz MA, Rühl M, Zorn H (2014) Food and feed enzymes. *Adv. Biochem. Eng. Biotechnol.* https://doi.org/10.1007/10_2013_235

32. Deckers M, Deforce D, Fraiture M-A, Roosens NHC (2020) Genetically Modified Micro-Organisms for Industrial Food Enzyme Production: An Overview. *Foods*. https://doi.org/10.3390/foods9030326

33. Steudler S, Werner A, Walther T (2019) It Is the Mix that Matters: Substrate-Specific Enzyme Production from Filamentous Fungi and Bacteria Through Solid-State Fermentation. *Adv. Biochem. Eng. Biotechnol.* https://doi.org/10.1007/10_2019_85

34. Berovic M (2019) Cultivation of Medicinal Mushroom Biomass by Solid-State Bioprocessing in Bioreactors. In: Steudler S, Werner A, Cheng JJ (eds) Solid State Fermentation: Research and Industrial Applications. *Springer International Publishing*, Cham. https://doi.org/10.1007/10_2019_89

35. Olempska-Beer ZS, Merker RI, Ditto MD, DiNovi MJ (2006) Food-processing enzymes from recombinant microorganisms--a review. *Regul. Toxicol. Pharmacol.* https://doi.org/10.1016/j.yrtph.2006.05.001

36. Spohner SC, Müller H, Quitmann H, Czermak P (2015) Expression of enzymes for the usage in food and feed industry with Pichia pastoris. *J. Biotechnol*. https://doi.org/10.1016/j.jbiotec.2015.01.027

37. Rieder L, Teuschler N, Ebner K, Glieder A (2019) Eukaryotic Expression Systems for Industrial Enzymes. 1.3. In: Industrial Enzyme Applications. *John Wiley & Sons, Ltd*. https://doi.org/10.1002/9783527813780.ch1_3

38. Donohoue PD, Barrangou R, May AP (2018) Advances in Industrial Biotechnology Using CRISPR-Cas Systems. *Trends Biotechnol.* https://doi.org/10.1016/j.tibtech.2017.07.007

39. Börner RA, Kandasamy V, Axelsen AM, Nielsen AT, Bosma EF (2019) Genome editing of lactic acid bacteria: opportunities for food, feed, pharma and biotech. *FEMS Microbiol. Lett*. https://doi.org/10.1093/femsle/fny291

40. Nødvig CS, Nielsen JB, Kogle ME, Mortensen UH (2015) A CRISPR-Cas9 System for Genetic Engineering of Filamentous Fungi. *PLoS One*. https://doi.org/10.1371/journal.pone.0133085

41. Zhang Y, Geary T, Simpson BK (2019) Genetically modified food enzymes: a review. *Curr. Opin. Food Sci.* https://doi.org/10.1016/j.cofs.2019.01.002

42. Debeuckelaere W (2015) Legislation on food additives, food enzymes and flavourings in the European Union. *Curr. Opin. Food Sci.* https://doi.org/10.1016/j.cofs.2015.12.001

43. Holtorf ML, Sachs G (2010) The new European Legislation on Food Additives, Flavourings and Enzymes. *Eur. Food Feed. Law Rev.* 5(3):173–178

44. European Parliament, European Council (2008) Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on food enzymes and amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/9

45. European Parliament, European Council (2008) Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives

46. European Parliament, European Council (2003) Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed

47. European Parliament, European Council Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004

48. Schulz S (2015) New EU Labeling Law: Omission of Food Additives and Enzymes from the List of New EU Labeling Law: Omission of Food Additives and Enzymes from the List of Ingredients under Regulation (EC) No. 1169/2011. *Eur. Food Feed. Law Rev*. 10(1):14–19

49. European Commision (2014) Gudiance document on criteria for categorization of food enzymes Online: https://ec.europa.eu/food/sites/food/files/safety/docs/fs_food-improvement-agents_enzymes-guidance-categorisation.pdf

50. MacGregor E, Janeček Š, Svensson B (2001) Relationship of sequence and structure to specificity in the α-amylase family of enzymes. *BBA*. https://doi.org/10.1016/s0167-4838(00)00302-2

51. McDonald AG, Boyce S, Tipton KF (2009) ExplorEnz: the primary source of the IUBMB enzyme list. *Nucleic Acids Res*. https://doi.org/10.1093/nar/gkn582

52. Davies GJ, Sinnott ML (2008) Sorting the diverse: the sequence-based classifications of carbohydrate-active enzymes. *Biomed. J.* https://doi.org/10.1042/BJ2008038

53. Dunn G (1974) A Model for Starch Breakdown in Higher Plants. *Phytochemistry*: https://doi.org/10.1016/0031-9422(74)80289-X

54. Repellin A, Båga M, Chibbar RN (2008) In vitro pullulanase activity of wheat (Triticum aestivum L.) limit-dextrinase type starch debranching enzyme is modulated by redox conditions. *J. Cereal Sci.* https://doi.org/10.1016/j.jcs.2007.04.015

55. Derde LJ, Gomand SV, Courtin CM, Delcour JA (2012) Hydrolysis of β-limit dextrins by α-amylases from porcine pancreas, Bacillus subtilis, Pseudomonas saccharophila and Bacillus stearothermophilus. *Food Hydrocoll.* https://doi.org/10.1016/j.foodhyd.2011.06.001

56. Goesaert H, Bijttebier A, Delcour JA (2010) Hydrolysis of amylopectin by amylolytic enzymes: level of inner chain attack as an important analytical differentiation criterion. *Carbohydr. Res.* https://doi.org/10.1016/j.carres.2009.11.011

57. Derde LJ, Gomand SV, Courtin CM, Delcour JA (2012) Characterisation of three starch degrading enzymes: thermostable β-amylase, maltotetraogenic and maltogenic α-amylases. *Food Chem.* https://doi.org/10.1016/j.foodchem.2012.05.031

58. Nonogaki H, Bassel GW, Bewley JD (2010) Germination—Still a mystery. *Plant Sci.* https://doi.org/10.1016/j.plantsci.2010.02.010

59. Ma Z, Bykova NV, Igamberdiev AU (2017) Cell signaling mechanisms and metabolic regulation of germination and dormancy in barley seeds. *Crop J.* https://doi.org/10.1016/j.cj.2017.08.007

60. Fincher GB (1989) Molecular and Cellular Biology Associated with Endosperm Mobilization in Germinating Cereal Grains. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* https://doi.org/10.1146/annurev.pp.40.060189.001513

61. Lemmens E, Moroni AV, Pagand J, Heirbaut P, Ritala A, Karlen Y, Lê K-A, den Broeck HC, Brouns FJ, Brier N, Delcour JA (2018) Impact of Cereal Seed Sprouting on Its Nutritional and Technological Properties. A Critical Review. *Compr. Rev. Food Sci. Food Saf.* https://doi.org/10.1111/1541-4337.12414

62. Olaerts H, Courtin CM (2018) Impact of Preharvest Sprouting on Endogenous Hydrolases and Technological Quality of Wheat and Bread. A Review. C*ompr. Rev. Food Sci. Food Saf.* https://doi.org/10.1111/1541-4337.12347

63. Mieog JC, Janeček Š, Ral J-P (2017) New insight in cereal starch degradation. Identification and structural characterization of four α-amylases in bread wheat. *Amylase*. https://doi.org/10.1515/amylase-2017-0004

64. Ral J-P, Whan A, Larroque O, Leyne E, Pritchard J, Dielen A-S, Howitt CA, Morell MK, Newberry M (2016) Engineering high α-amylase levels in wheat grain lowers Falling Number but improves baking properties. *Plant Biotechnol. J.* https://doi.org/10.1111/pbi.12390

65. Sun Z, Henson CA (1991) A quantitative Assessment of the Importance of Barley Seed α-Amylase, β-Amylase, Debranching Enzyme and α-Glucosidase in Starch Dedgadation. *Arch. Biochem. Biophys.* https://doi.org/10.1016/0003-9861(91)90299-x

66. Aoki N, Scofield GN, Wang X-D, Offler CE, Patrick JW, Furbank RT (2006) Pathway of sugar transport in germinating wheat seeds. *Plant Physiol.* https://doi.org/10.1104/pp.106.082719

67. Ziegler P (1999) Cereal Beta-Amylases. *J. Cereal Sci.* https://doi.org/10.1006/jcrs.1998.0238

68. Barrero JM, Mrva K, Talbot MJ, White RG, Taylor J, Gubler F, Mares DJ (2013) Genetic, hormonal, and physiological analysis of late maturity α-amylase in wheat. *Plant Physiol.* https://doi.org/10.1104/pp.112.209502

69. Mares DJ, Mrva K (2014) Wheat grain preharvest sprouting and late maturity alpha-amylase. *Planta*. https://doi.org/10.1007/s00425-014-2172-5

70. Newberry M, Zwart AB, Whan A, Mieog JC, Sun M, Leyne E, Pritchard J, Daneri-Castro SN, Ibrahim K, Diepeveen D, Howitt CA, Ral J-PF (2018) Does Late Maturity Alpha-Amylase Impact Wheat Baking Quality? *Front. Plant Sci.* https://doi.org/10.3389/fpls.2018.01356

71. Mares D, Mrva K (2008) Late-maturity α-amylase: Low falling number in wheat in the absence of preharvest sprouting. *J. Cereal Sci.* https://doi.org/10.1016/j.jcs.2007.01.00

72. Heiss AG, Azorín MB, Antolín F, Kubiak-Martens L, Marinova E, Arendt EK, Biliaderis CG, Kretschmer H, Lazaridou A, Stika H-P, Zarnkow M, Baba M, Bleicher N, Ciałowicz KM, Chłodnicki M, Matuschik I, Schlichtherle H, Valamoti SM (2020) Mashes to Mashes, Crust to Crust. Presenting a novel microstructural marker for malting in the archaeological record. *PLoS One*. https://doi.org/10.1371/journal.pone.0231696

73. Struyf N, van der Maelen E, Hemdane S, Verspreet J, Verstrepen KJ, Courtin CM (2017) Bread Dough and Baker's Yeast. An Uplifting Synergy. *Compr. Rev. Food Sci. Food Saf.* https://doi.org/10.1111/1541-4337.12282

74. Struyf N, Verspreet J, Verstrepen KJ, Courtin CM (2017) Investigating the impact of α-amylase, α-glucosidase and glucoamylase action on yeast-mediated bread dough fermentation and bread sugar levels. *J. Cereal Sci*. https://doi.org/10.1016/j.jcs.2017.03.013

75. Barrera GN, Tadini CC, León AE, Ribotta PD (2016) Use of alpha-amylase and amyloglucosidase combinations to minimize the bread quality problems caused by high levels of damaged starch. *J. Food Sci. Technol.* https://doi.org/10.1007/s13197-016-2337-2

76. Struyf N, Verspreet J, Courtin CM (2016) The effect of amylolytic activity and substrate availability on sugar release in non-yeasted dough. *J. Cereal Sci.* https://doi.org/10.1016/j.jcs.2016.02.016

77. Cho IH, Peterson DG (2010) Chemistry of bread aroma. A review. *Food Sci Biotechnol*. https://doi.org/10.1007/s10068-010-0081-3

78. Purlis E (2010) Browning development in bakery products – A review. *J. Food Eng.* https://doi.org/10.1016/j.jfoodeng.2010.03.008

79. Pozo-Bayón MA, Guichard E, Cayot N (2006) Flavor Control in Baked Cereal Products. *Food Rev. Int*. https://doi.org/10.1080/87559120600864829

80. Anja Niehues Birch, Mikael Agerlin Petersen, Åse Solvej Hansen (2014) Aroma of Wheat Bread Crumb. *Cereal Chem* https://doi.org/10.1094/CCHEM-06-13-0121-RW

81. Pico J, Bernal J, Gómez M (2015) Wheat bread aroma compounds in crumb and crust. A review. *Food Res. Int*. https://doi.org/10.1016/j.foodres.2015.05.051

82. Antonia Martínez-Anaya M (1996) Enzymes and Bread Flavor. *J. Agric. Food Chem.* https://doi.org/10.1021/jf960020d

83. Błaszczak W, Sadowska J, Rosell CM, Fornal J (2004) Structural changes in the wheat dough and bread with the addition of alpha-amylases. *Eur Food Res Technol*. https://doi.org/10.1021/jf960020d

84. Goesaert H, Leman P, Bijttebier A, Delcour JA (2009) Antifirming effects of starch degrading enzymes in bread crumb. *J. Agric. Food Chem.* https://doi.org/10.1021/jf803058v

85. Palacios HR, Schwarz PB, D'Appolonia BL (2004) Effect of alpha-amylases from different sources on the retrogradation and recrystallization of concentrated wheat starch gels: relationship to bread staling. *J. Agric. Food Chem*. https://doi.org/10.1021/jf030377z

86. Gray JA, Bemiller JN (2003) Bread Staling: Molecular Basis and Control. *Compr. Rev. Food Sci. Food Saf*. https://doi.org/10.1111/j.1541-4337.2003.tb00011.x

87. Goesaert H, Slade L, Levine H, Delcour JA (2009) Amylases and bread firming – an integrated view. *J. Cereal Sci.* https://doi.org/10.1016/j.jcs.2009.04.010

88. Ribotta PD, Le Bail A (2007) Thermo-physical assessment of bread during staling. *LWT*. https://doi.org/10.1016/j.lwt.2006.03.023

89. Hug-Iten S, Fischer F., Conde-Petit B (2003) Staling of Bread: Role of Amylose and Amylopectin and Influence of Starch-Degrading Enzymes. *Cereal Chem*. https://doi.org/10.1094/CCHEM.2003.80.6.654

90. Bosmans GM, Lagrain B, Ooms N, Fierens E, Delcour JA (2013) Biopolymer interactions, water dynamics, and bread crumb firming. *J. Agric. Food Chem.* https://doi.org/10.1021/jf4010466

91. Hug-Iten S, Fischer F, Conde-Petit B (2001) Structural Properties of Starch in Bread and Bread Model Systems: Influence of an Antistaling alpha-Amylase. *Cereal Chem*. https://doi.org/10.1094/CCHEM.2001.78.4.421

92. Defloor I, Delcour JA (1999) Impact of maltodextrins and antistaling enzymes on the differential scanning calorimetry staling endotherm of baked bread doughs. *J. Agric. Food Chem.* https://doi.org/10.1021/jf9806356

93. Bosmans GM, Lagrain B, Fierens E, Delcour JA (2013) Impact of amylases on biopolymer dynamics during storage of straight-dough wheat bread. *J. Agric. Food Chem.* https://doi.org/10.1021/jf402021g

94. Bae W, Lee SH, Yoo S-H, Lee S (2014) Utilization of a maltotetraose-producing amylase as a whole wheat bread improver: dough rheology and baking performance. *J. Food Sci.* https://doi.org/10.1111/1750-3841.12538

95. Lagrain B, Leman P, Goesaert H, Delcour JA (2008) Impact of thermostable amylases during bread making on wheat bread crumb structure and texture. *Food Res. Int.* https://doi.org/10.1016/j.foodres.2008.07.006

96. Amigo JM, Del Olmo Alvarez A, Engelsen MM, Lundkvist H, Engelsen SB (2016) Staling of white wheat bread crumb and effect of maltogenic α-amylases. Part 1. Spatial distribution and kinetic modeling of hardness and resilience. *Food Chem.* https://doi.org/10.1016/j.foodchem.2016.02.162

97. Zhang L, Chen XD, Boom RM, Schutyser MAI (2017) Thermal inactivation kinetics of β-galactosidase during bread baking. *Food Chem*. https://doi.org/10.1016/j.foodchem.2017.01.010

98. Perdana J, Fox MB, Schutyser M, Boom RM (2012) Enzyme inactivation kinetics. Coupled effects of temperature and moisture content. *Food Chem*. https://doi.org/10.1016/j.foodchem.2011.12.080

99. Samborska K, Guiavarc'h Y, van Loey A, Hendrickx M (2005) The influence of moisture content on the thermostability of Aspergillus oryzae α-amylase. *Enzyme Microb. Technol.* https://doi.org/10.1016/j.enzmictec.2004.06.017

100. Daniel RM, Danson MJ, Eisenthal R, Lee CK, Peterson ME (2008) The effect of temperature on enzyme activity: new insights and their implications. Extremophiles: life under extreme conditions 12(1):51–59

101. Daniel RM, Danson MJ, Eisenthal R, Lee CK, Peterson ME (2008) The effect of temperature on enzyme activity: new insights and their implications. *Extremophiles.* https://doi.org/10.1007/s00792-007-0089-7

102. Alikhajeh J, Khajeh K, Ranjbar B, Naderi-Manesh H, Lin YH, Liu E, Guan HH, Hsieh YC, Chuankhayan P, Huang YC, Jeyaraman J, Liu MY, Chen CJ (2010) Structure of Bacillus

amyloliquefaciens alpha-amylase at high resolution: implications for thermal stability. *Acta Crystallogr. F 66.* https://doi.org/10.1107/S1744309109051938

103. Reichenberger K, Luz A, Seitl I, Fischer L (2020) Determination of the Direct Activity of the Maltogenic Amylase from Geobacillus stearothermophilus in White Bread. *Food Anal. Methods*. https://doi.org/10.1007/s12161-019-01673-7

104. Yu N, Laurenz R, Siler L, Ng PKW, Souza E, Lewis JM (2015) Evaluation of α-amylase activity and falling number around maturity for soft white and soft red wheat varieties in Michigan. *Cereal Res. Commun.* https://doi.org/10.1556/0806.43.2015.026

105. Arbeitsgemeinschaft Getreideforschung e.V. (ed) (2016) Standard-Methoden für Getreide, Mehl und Brot mit allen aktuellen ICC-Standards, 8.th edn. Verlag Moritz Schäfer, Detmold

106. Delwiche SR, Vinyard BT, Bettge AD (2015) Repeatability Precision of the Falling Number Procedure Under Standard and Modified Methodologies. *Cereal Chem.* https://doi.org/10.1094/CCHEM-07-14-0156-R

107. Kiszonas AM, Engle DA, Pierantoni LA, Morris CF (2018) Relationships between Falling Number, α-amylase activity, milling, cookie, and sponge cake quality of soft white wheat. *Cereal Chem*. https://doi.org/10.1002/cche.10041

108. Codina GG, Mironeasa S, Mironeasa C (2012) Variability and relationship among Mixolab and Falling Number evaluation based on influence of fungal α-amylase addition. *J. Sci. Food Agric.* https://doi.org/10.1002/jsfa.5603

109. Williams RM, Diepeveen DA, Evans FH (2019) Using big data to predict the likelihood of low falling numbers in wheat. *Cereal Chem.* https://doi.org/10.1002/cche.10140

110. Bisswanger H (2014) Enzyme assays. *Perspect Sci.* https://doi.org/10.1016/j.pisc.2014.02.005

111. Visvanathan R, Qader M, Jayathilake C, Jayawardana BC, Liyanage R, Sivakanesan R (2020) Critical review on conventional spectroscopic α-amylase activity detection methods: merits, demerits, and future prospects. *J. Sci. Food Agric.* https://doi.org/10.1002/jsfa.10315

112. McCleary BV, Sheehan H (1987) Measurement of cereal α-amylase: A new assay procedure. *J. Cereal Sci*. https://doi.org/10.1016/S0733-5210(87)80061-9

113. McCleary BV, Codd R (1989) Measurement of β-amylase in cereal flours and commercial enzyme preparations. *J. Cereal Sci*. https://doi.org/10.1016/S0733-5210(89)80018-9

114. McCleary BV, Mangan D, McKie V, Cornaggia C, Ivory R, Rooney E (2014) Colourimetric and fluorometric substrates for measurement of pullulanase activity. *Carbohydr. Res*. https://doi.org/10.1016/j.carres.2014.04.014

115. McCleary BV, Sheehan H (1987) Measurement of cereal α-amylase: A new assay procedure. *J. Cereal Sci*. https://doi.org/10.1016/S0733-5210(87)80061-9

116. McCleary BV, McNally M, Monaghan D (2002) Measurement of a-Amylase activity in white wheat flour, milled malt, and microbial enzyme preparations, using the ceralpha assay: collaborative study. *J AOAC Int*. https://doi.org/10.1093/jaoac/85.5.1096

117. Toutounji MR, van Leeuwen MP, Oliver JD, Shrestha AK, Castignolles P, Gaborieau M (2015) Quantification of sugars in breakfast cereals using capillary electrophoresis. *Carbohydr. Res*. https://doi.org/10.1016/j.carres.2015.03.008

118. Liu Z, Rochfort S (2015) Identification and quantitative analysis of oligosaccharides in wheat flour using LC–MS. *J. Cereal Sci.* https://doi.org/10.1016/j.jcs.2015.03.009

119. Ruiz-Matute AI, Brokl M, Soria AC, Sanz ML, Martínez-Castro I (2010) Gas chromatographic–mass spectrometric characterisation of tri- and tetrasaccharides in honey. *Food Chem*. https://doi.org/10.1016/j.foodchem.2009.10.050

120. Al-Mhanna NM, Huebner H, Buchholz R (2018) Analysis of the Sugar Content in Food Products by Using Gas Chromatography Mass Spectrometry and Enzymatic Methods. *Foods*. https://doi.org/10.3390/foods7110185

121. Corradini C, Cavazza A, Bignardi C (2012) High-Performance Anion-Exchange Chromatography Coupled with Pulsed Electrochemical Detection as a Powerful Tool to Evaluate Carbohydrates of Food Interest: Principles and Applications. *International J Carbohydr Chem*. https://doi.org/10.1155/2012/487564

122. Ispiryan L, Heitmann M, Hoehnel A, Zannini E, Arendt EK (2019) Optimization and Validation of an HPAEC-PAD Method for the Quantification of FODMAPs in Cereals and Cereal-Based Products. *J. Agric. Food Chem*. https://doi.org/10.1021/acs.jafc.9b00382

123. Langemeier JM, Rogers DE (1995) Rapid Method for Sugar Analysis of doughs and Baked products. *Cereal Chem*. 72(4):349–351

124. Pico J, Martínez MM, Martín MT, Gómez M (2015) Quantification of sugars in wheat flours with an HPAEC-PAD method. *Food Chem.* https://doi.org/10.1016/j.foodchem.2014.10.103

125. Aebersold R, Mann M (2003) Mass spectrometry-based proteomics. *Nature*. https://doi.org/10.1038/nature01511

126. Cox J, Mann M (2011) Quantitative, high-resolution proteomics for data-driven systems biology. *Annu. Rev. Biochem*. https://doi.org/10.1146/annurev-biochem-061308-093216

127. Zhang Y, Fonslow BR, Shan B, Baek M-C, Yates JR (2013) Protein analysis by shotgun/bottom-up proteomics. *Chem. Rev*. https://doi.org/10.1021/cr3003533

128. Hu A, Noble WS, Wolf-Yadlin A (2016) Technical advances in proteomics: new developments in data-independent acquisition. *F1000research.* https://doi.org/10.12688/F1000research.7042.1

129. The UniProt Consortium (2019) UniProt: a worldwide hub of protein knowledge. *Nucleic Acids Res.* https://doi.org/10.1093/nar/gky1049

130. Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* https://doi.org/10.1038/nbt.1511

131. Ludwig C, Gillet L, Rosenberger G, Amon S, Collins BC, Aebersold R (2018) Data-independent acquisition-based SWATH-MS for quantitative proteomics. A tutorial. *Mol. Syst. Biol.* https://doi.org/10.15252/msb.20178126

132. Schwanhäusser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, Chen W, Selbach M (2011) Global quantification of mammalian gene expression control. *Nature.* https://doi.org/10.1038/nature10098

133. Geisslitz S, Ludwig C, Scherf KA, Koehler P (2018) Targeted LC-MS/MS Reveals Similar Contents of α-Amylase/Trypsin-Inhibitors as Putative Triggers of Nonceliac Gluten Sensitivity in All Wheat Species except Einkorn. *J. Agric. Food Chem.* https://doi.org/10.1021/acs.jafc.8b04411

134. Uhr L, Buchholz T, Homann T, Huschek G, Rawel HM (2014) Targeted proteomics-based analysis of technical enzymes from fungal origin in baked products. *J. Cereal Sci.* https://doi.org/10.1016/j.jcs.2014.04.007

135. Uhr L, Wieland P, Homann T, Huschek G, Rawel HM (2016) Identification and LC–MS/MS-based analyses of technical enzymes in wheat flour and baked products. *Eur Food Res Techno*l. https://doi.org/10.1007/s00217-015-2536-5

136. Picariello G, Di Stasio L, Mamone G, Iacomino G, Venezia A, Iannaccone N, Ferranti P, Coppola R, Addeo F (2018) Identification of enzyme origin in dough improvers. DNA-based and proteomic approaches. *Food Res. Int*. https://doi.org/10.1016/j.foodres.2017.10.046

137. Prandi B, Lambertini F, Varani M, Faccini A, Suman M, Leporati A, Tedeschi T, Sforza S (2020) Assessment of Enzymatic Improvers in Flours Using LC-MS/MS Detection of Marker Tryptic Peptides. *J. Am. Soc. Mass Spectrom.* https://doi.org/10.1021/jasms.9b00109

138. Ainsworth C, Clark J, Balsdon J (1993) Expression, organisation and structure of the genes encoding the waxy protein (granule-bound starch synthase) in wheat. *Plant Mol. Biol.* https://doi.org/10.1007/BF00038996

139. Brzozowski AM, Lawson DM, Turkenburg JP, Bisgaard-Frantzen H, Svendsen A, Borchert TV, Dauter Z, Wilson KS, Davies GJ (2000) Structural analysis of a chimeric bacterial alpha-amylase. High-resolution analysis of native and ligand complexes. *Biochemistry.* https://doi.org/10.1021/bi0000317

140. Jayaram VB, Cuyvers S, Lagrain B, Verstrepen KJ, Delcour JA, Courtin CM (2013) Mapping of Saccharomyces cerevisiae metabolites in fermenting wheat straight-dough reveals succinic acid as pH-determining factor. Food Chem. https://doi.org/10.1016/j.foodchem.2012.08.039

141. Holmes JT, Hoseney RC (1987) Chemical Leavening: Effect of pH and Certain Ions in Breadmaking Properties. *Cereal Chem.* 46(5):343–348

142. Lin W, Lineback DR (1990) Changes in Carbohydrate Fractions in Enzyme-Supplemented Bread and the Potential Relationship to Staling. *Starke*. https://doi.org/10.1002/star.19900421005

143. van der Sman R (2016) Sugar and polyol solutions as effective solvent for biopolymers. *Food Hydrocoll*. https://doi.org/10.1016/j.foodhyd.2015.12.001

144. van der Sman R, Mauer LJ (2019) Starch gelatinization temperature in sugar and polyol solutions explained by hydrogen bond density. *Food Hydrocoll.* https://doi.org/10.1016/j.foodhyd.2019.03.034

145. van der Maarel MJ, van der Veen B, Uitdehaag JC, Leemhuis H, Dijkhuizen L (2002) Properties and applications of starch-converting enzymes of the α-amylase family. *J. Biotechnol.* https://doi.org/10.1016/S0168-1656(01)00407-2

146. Bijttebier A, Goesaert H, Delcour JA (2007) Temperature impacts the multiple attack action of amylases. *Biomacromolecules.* https://doi.org/10.1021/bm060784u

6. Further research

This work is based on protein identification and quantitation, the determination of amylase activity and knowledge about sugar release in wheat bread. Therefore, several existing methods were modified and new methods were established. Within this process a number of new research questions arose.

A higher number of samples of amylase preparations would result in a larger amount of data, but for an even more comprehensive assessment of the mechanisms of exogenous amylase preparations in wheat bread baking, it is not only sufficient to increase the number of samples, but rather to perform additional analytical approaches.

To provide more knowledge on amylase activity and sugar release in wheat bread, the dosage of amylase preparations could be varied in baking experiments. In this way the impact of specific preparations related to the dosage could be investigated. Further, a minimum dosage of the preparations as well as the effect of overdosing the preparations could be determined. Additionally, pure analytical grade amylases could be applied to get deeper insights of the impact of the preparation matrix on activity or sugar release. Complementing the sample set, the analysis of different spots inside the bread crumb could be conducted. Thus, a more general statement on the distribution of residual activity could be made.

To make the baking experiments more realistic in the sense of production of baked goods in the industry and in the craft sector, yeast should be included in the baking recipe. With this modification, endogenous and exogenous amylases must deal with the whole microbiome of wheat bread. Thus, the impact of the microbiome on enzyme activity could be determined and the results directly applied to commercially produced wheat bread. In order to make a statement about a technological function in the final product and thus provide an answer to the question of whether the enzyme needs to be declared or not, it would still be necessary to analyze changes in the crumb texture of bread with residual amylase activity during storage.

A future challenge regarding the identification and quantitation of amylases in wheat bread baking is their analysis not only in the preparations but in the bread itself. With the discovery-driven proteomics method established in this study, knowledge about peptides generated during tryptic digest was established. Via the selection of marker peptides for each individual amylase it would be possible to establish a targeted detection method for the amylases. Due to their low dosage of only a few mg per kg, the sensitivity of the method must be increased. This could be achieved by combining extraction and tryptic digestion in one step and by an enrichment of the targeted amylase in the solution for analysis. For a targeted analysis, a state-of-the-art high resolution QQQ-MS system or an Orbitap system for discovery-driven analysis is likely to increase sensitivity.

7. Summary

Several different amylases are commonly added during the manufacturing of wheat bread. They are intended to be processing aids to help improve the quality of wheat bread via their impact on bread volume, crust color, bread flavor and staling. Until now, it was assumed that exogenous amylases are completely denatured by heat during the baking process and therefore no longer have any residual activity in the bread. Little is known so far about the mechanisms of residual amylase activity, i.e., about amylases showing residual activity and their influence on sugar release in wheat breads during storage.

In this work, commercially available amylase preparations intended for use in wheat bread making containing α-amylase, maltotetraogenic amylase and maltogenic α-amylase from different microbial sources were investigated. To get deeper insights into the mechanisms of these exogenous amylases, we categorized the work into three parts. In the first part we identified the amylase causing the amylolytic effect of the amylase preparations by proteomics techniques, in the second part we detected the residual activity resulting from the preparations in bread crumb and in the third part we analyzed the dynamics of sugar release in wheat bread crumb during storage.

We identified one amylase in each of the two bacterial α-amylase preparations, and two proteins in each of the fungal α-amylase preparations one α-amylase and one glucoamylase. Further analysis showed that the α-amylase D9J2M5 was the main protein responsible for the amylolytic activity of the fungal preparations. In the maltotetraogenic preparations P22963 from *Pelomonas saccharophila* and in the maltogenic α-amylase P19531 from *Geobacillus stearothermophilus* were identified to be the proteins responsible for the amylolytic activity. Within the framework of the discovery-driven proteomics approach, we investigated the proportion of amylase present in each preparation. Wide variations in the proportion of amylases were found to be present over the entire sample set, ranging from 5% to 97%.

The α-amylase of fungal origin showed poor temperature stability in the baking experiments, and consequently no residual activity was measured. The *Bacillus amyloliquefaciens* α-amylase was the only amylase showing a very low residual activity in wheat bread crumb compared to the remaining α-amylases with no detectable activity.

All types of maltotetraogenic amylase and maltogenic α-amylases preparations showed residual activity in wheat bread crumb. The relative residual activity of maltotetraogenic amylase with 1.1% and 1.9% was lower than that of maltogenic α-amylase preparations which had activities ranging from 13.9% up to 45.5%.

Regarding the sugar spectra of the model breads with α-amylase addition, we found that despite a lack of residual activity, changes in the content of mono-, di-, and oligosaccharides

were present. These changes might be traced back to retrogradation and a modified extractability of sugars due to changes related to hydrogen bonds within the starch polymer.

For all preparations containing maltotetraogenic amylase and maltogenic α-amylase changes in the sugar spectrum were detected. A direct correlation between the level of residual activity and the amount of sugars formed during storage was not found. All maltooligosaccharides formed during storage are described as hydrolysis products of maltotetraogenic amylase and maltogenic α-amylase in literature. Thus, they are likely to be hydrolysis products related to the detected residual amylase activity.

Whether the detected residual activity is related to characteristics of bread staling cannot be answered by the findings of this work and this has to be clarified in further studies.

The scientific contribution of this work was the elucidation of basic mechanisms of exogenous amylases regarding their residual activity and the dynamics of sugar release during storage. Therefore, fundamental knowledge about the detailed composition of commonly available amylase preparations was generated. These findings were connected to the residual activity of the respective amylase and further, it was shown that sugar concentrations during storage are affected when using these preparations. To sum up, the experimental design including a proteomics-based approach coupled with the detection of residual activity and the quantitation of sugars during storage allowed a comprehensive observation of the activity of exogenous amylases and their impact on the sugar pattern in wheat bread during storage.

8. Zusammenfassung

Bei der Herstellung von Weizenbrot werden oft mehrere verschiedene Amylasen zugesetzt. Sie sollen sich als Verarbeitungshilfsstoffe positiv auf die Brotqualität auswirken. Dies geschieht durch ihren Effekt auf das Brotvolumen, die Krustenfarbe und den Brotgeschmack, sowie auf das Altbackenwerden. Bisher wurde davon ausgegangen, dass exogene Amylasen durch die Temperatureinwirkung während des Backprozesses vollständig denaturiert werden und somit keine Restaktivität mehr im Brot haben. Über die Mechanismen einer Restaktivität von Amylasen und deren Einfluss auf die Zuckerfreisetzung in Weizenbroten während der Lagerung, ist bisher wenig bekannt.

Im Rahmen dieser Arbeit wurden kommerziell erhältliche Amylasepräparate für die Verwendung in der Weizenbrotproduktion untersucht, die α-Amylase, maltotetragene Amylase und maltogene α-Amylase aus verschiedenen mikrobiellen Quellen enthielten. Um genauere Einblicke in die Mechanismen dieser exogenen Amylasen zu erhalten, wurde die Arbeit in drei Teilbereiche gegliedert. Im ersten Teil identifizierten wir mit Hilfe von Proteomics-Techniken diejenige Amylase, die den amylolytischen Effekt der Amylasepräparate verursacht, im zweiten Teil wurde die aus den Präparaten resultierende Restaktivität in der Brotkrume nachgewiesen und im dritten Teil wurde die Dynamik der Zuckerfreisetzung in der Weizenbrotkrume während der Lagerung analysiert.

Für die beiden bakteriellen α-Amylase-Präparate wurde jeweils eine Amylase identifiziert; für die fungale α-Amylase jeweils zwei verschiedene Amylasen. In den fungalen Präparaten wurde eine α-Amylase und eine Glucoamylase nachgewiesen. Weitere Analysen zeigten, dass die α-Amylase D9J2M5 das Hauptprotein ist, das für die amylolytische Aktivität der Pilzpräparate verantwortlich ist. In den maltotetragenen Präparaten wurde P22963 aus *Pelomonas saccharophila* und in der maltogenen α-Amylase P19531 aus *Geobacillus stearothermophilus* als das für die amylolytische Aktivität verantwortliche Protein identifiziert. Im Rahmen des Discovery-Driven Proteomics-Ansatzes wurde untersucht, wie groß der Anteil der Amylase in den einzelnen Präparaten ist. Es wurde festgestellt, dass über die Gesamtheit des Probensatzes der Amylaseanteil 5% bis 97% betrug.

Die fungale α-Amylase zeigte in den Backversuchen eine niedrige Temperaturstabilität, so dass für sie keine Restaktivität gemessen werden konnte. Die α-Amylase von *Bacillus amyloliquefaciens* war die einzige Amylase aus der Gruppe der α-Amylasen, die eine sehr geringe Restaktivität in der Weizenbrotkrume aufwies.

Alle untersuchten maltotetragenen Amylasepräparate und maltogenen α-Amylasepräparate zeigten Restaktivität in der Weizenbrotkrume. Dabei war die relative Restaktivität der

maltotetragenen Amylase mit 1,1% und 1,9% geringer als die der maltogenen α-Amylase-Präparate, die von 13,9% bis 45,5% reichte.

Hinsichtlich der Zuckerspektren der Modellbrote mit α-Amylasezusatz wurde festgestellt, dass trotz fehlender Restaktivität Veränderungen im Gehalt an Mono-, Di- und Oligosacchariden vorhanden waren. Diese Veränderungen könnten auf eine Retrogradation und eine veränderte Extrahierbarkeit von Zuckern aufgrund von Veränderungen im Zusammenhang mit Wasserstoffbrückenbindungen innerhalb des Stärkepolymers zurückzuführen sein.

Bei allen Präparaten, die maltotetragene Amylase und maltogene α-Amylase enthielten, wurden Veränderungen im Zuckerspektrum festgestellt. Eine direkte Korrelation zwischen der Höhe der Restaktivität und der Menge der während der Lagerung gebildeten Zucker wurde nicht gefunden. Alle während der Lagerung gebildeten Maltooligosaccharide werden in der Literatur als Hydrolyseprodukte der maltotetragenen Amylase und der maltogenen α-Amylase beschrieben. Es ist somit höchstwahrscheinlich, dass es sich um Hydrolyseprodukte handelt, die aus der Amylase Restaktivität resultieren.

Ob die detektierte Restaktivität mit Mechanismen der Brotalterung zusammenhängt, kann anhand der Ergebnisse dieser Arbeit nicht beantwortet werden und muss in weiteren Untersuchungen geklärt werden.

Der wissenschaftliche Beitrag dieser Arbeit ist die Aufklärung grundlegender Mechanismen von exogenen Amylasen hinsichtlich ihrer Restaktivität und der Dynamik der Zuckerfreisetzung während der Lagerung. Dazu wurden grundlegende Erkenntnisse über die detaillierte Zusammensetzung von handelsüblichen Amylasepräparaten gewonnen. Diese Erkenntnisse wurden mit der Restaktivität der jeweiligen Amylase in Verbindung gebracht und weiterhin wurde gezeigt, dass die Zuckerkonzentrationen während der Lagerung durch Verwendung dieser Präparate beeinflusst werden. Der Versuchsaufbau mit einem Proteomics-basierten Ansatz, gekoppelt mit der Detektion der Restaktivität und der Quantifizierung der Zucker während der Lagerung, ermöglicht somit eine umfassende Betrachtung der Aktivität exogener Amylasen und deren Einfluss auf das Zuckerspektrum in Weizenbrot während der Lagerung.