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Optimization of Lipase Application by Means of Activity-Guided Lipid Fractionation

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Table of contents

Abbreviations III

1. Introduction 1

1.1 Wheat flour and breadmaking 1

1.1.1 Wheat flour 1

1.1.2 Wheat flour constituents 2

1.1.2.1 Starch 2

1.1.2.2 Non-starch polysaccharides 4

1.1.2.3 Proteins 4

1.1.3 Wheat flour lipids 7

1.2 Surfactants 15

1.2.1 Surface-active molecules and interfaces 16

1.2.1 Classification 18

1.2.2 Commercial surfactants for the baking industry 18

1.2.2.1 Mono- and diglycerides 18

1.2.2.2 Diacetyl tartaric acid esters of monoglycerides 19

1.2.2.3. Stearoyl-2-lactylate 20

1.2.3 Interaction between surfactants and wheat dough components 21

1.3 Lipases for baking applications 23

1.3.1 Lipases 23

1.3.2 Lipase activity assays 26

1.3.3 Baking lipases 27

1.3.4 Functionality of lipases in the production of wheat bread 29

1.4 Methods to isolate lipid classes and their analytical characterization 32

1.4.1 Isolation of non-polar and polar lipids from wheat flour 32

1.4.2 Fractionation and analytical characterization of wheat flour lipids 34

1.5 Methods to evaluate the functional effects of lipid-mixtures 36

1.5.1 Effects on the mixing properties 36

1.5.2 Developed dough and gluten 37

1.5.3 Confocal laser scanning microscopy (CLSM) 38

1.5.4 Baking activity 38

1.5.5 Reconstitution baking tests 39

1.5.6 Crumb softness and anti-staling effect 40

1.6 Research requirements in the field of lipases in breadmaking 40

1.7 References 42

2. Aim of the work 54

3. Results 55

3.1 Lipases in wheat breadmaking: analysis and functional effects of lipid reaction products 55

3.2 Reconstitution baking tests with defatted wheat flour are suitable to determine the functional effects of lipase-treated wheat lipids 71

3.3 Relationships between lipase-treated wheat lipid classes and their functional effects in wheat breadmaking 82

4. General discussion 98

4.1 References 110

5. Future research 111

6. Summary 112

7. Zusammenfassung 115

Abbreviations

AACCI	American Association of Cereal Chemists International
AOCS	American Oil Chemists Society
ASG	acylated sterol glucosides
BU	Brabender Units
CBR	cerebrosides
CER	ceramides
CLSM	confocal laser scanning microscopy
DG	diglycerides
DATEM	diacetyl tartaric acid esters of mono- and diglycerides
DGDG	digalactosyl diglycerides
DGMG	digalactosyl monoglycerides
dm	dry matter
ELSD	evaporative light scattering detector
FFA	free fatty acids
GC-FID	gas chromatography with a flame ionization detector
HLB	hydrophilic lipophilic balance
HMW-GS	high-molecular-weight glutenin subunits
HPLC	high-performance liquid chromatography
HPTLC	high performance thin layer chromatography
LC-MS	liquid chromatography–mass spectrometry
LMW-GS	low-molecular-weight glutenin subunits
LPC	lysophosphatidyl choline
LPE	lysophosphatidyl ethanolamine
LPI	lysophosphatidyl inositol

Abbreviations

MBT	micro-baking test
MG	monoglycerides
MGDG	monogalactosyl diglycerides
MGMG	monogalactosyl monoglycerides
MRMT	micro-rapid-mix test
MS	mass spectrometry
NALPE	N-acyl-lysophosphatidyl ethanolamine
NAPE	N-acyl-phosphatidyl ethanolamine
NMR	nuclear magnetic resonance
NP	normal-phase
PA	phosphatidic acid
PC	phosphatidyl choline
PE	phosphatidyl ethanolamine
PI	phosphatidyl inositol
PS	phosphatidyl serine
PINs	puroindolines
RP-HPLC	reversed-phase high-performance liquid chromatography
RSD	relative standard deviation
SD	standard deviation
SG	sterol glucosides
SSL	sodium stearoyl lactylate
TG	triglycerides
WSB	water-saturated *n*-butanol

1. Introduction

1.1 Wheat flour and breadmaking

1.1.1 Wheat flour

Bread has a long history and undoubtedly a long future. Breadmaking is a centuries-old traditional craft, practiced in any country capable of growing or importing wheat (Cauvain, 2012a). A huge range of bread products has evolved over the centuries. In some countries the nature of breadmaking has retained its traditional form while in others is has changed dramatically. The globalization of wheat trading was probably the first step in the globalization of baking including the access to new wheat varieties (Cauvain, 2012b). Consumers may want different products, but consistent quality remains a prerequisite. The huge market for bread and bakery products is still growing and the demand for good quality bread is undiminished.

Wheat (*Triticum aestivum* L. and other *triticum* species) and rye (*Secale cereale* L.) are the types of grain traditionally associated with breadmaking. Wheat flour provides unique properties amongst cereal flours and is, therefore, the basis for the successful production of bread. Wheat is grown in most parts of the world, from near-arctic to near-equatorial latitudes. It is the most important crop among the cereals by area planted and is followed in importance by corn, barley, and sorghum (FAO, 2009). International wheat breeding for increased productivity has been claimed to be the major reason for an additional 14 - 41 million tons of wheat per year (Souci et al., 2011). Wheat flour products are staple foods world-wide. Especially bread products are providing the majority of the daily carbohydrate, protein and vitamin B requirements. The wheat production world-wide was 726 million tons in the year 2014. In Germany, the production was 25 million tons that is equal to 3.5% of the world-wide production (Faostat, 2014). The production of wheat increased by 69% from 1961 to 2013. The trend shows that the global demand for wheat will further increase because of increase of population and prosperity.

Wheat flour is a result of the milling process of wheat grain. Various types of wheat flour with different compositions can be obtained by specific milling processes. The technological challenge of separating the three main parts of the wheat kernel (endosperm, germ and bran) is complex because of the kernel shape, the crease, and the aleurone layer. The amount of flour recovered, while its qualities are kept at specified levels, is usually expressed as flour

extraction. The different available flour types are labelled according to the ash mass that remains after a sample is incinerated in a laboratory oven based on the ICC method 104/1 (ICC 1990). The higher the ash content the higher the content of protein, fibres, minerals and vitamins. The main constituents of wheat flour are starch (70-75%), protein (10-12%), and water (14%). Among the minor components, non-starch polysaccharides (2-3%, arabinoxylans) and lipids (2%) have some relevance in breadmaking (Buck et al., 2007). The quality of wheat flour is essentially dependent on three factors: the individual genetic predisposition of wheat cultivar, the agricultural practices used during cultivation, and the environmental effects the plant was exposed to (Wieser & Seilmeier, 1998). These exogenous and endogenous factors have great influence on the individual constituents of wheat flour and, therefore, on its overall baking quality. These properties can be influenced with additives and in this way fluctuating wheat quality can be compensated for.

1.1.2 Wheat flour constituents

1.1.2.1 Starch

The major constituent of wheat flour is the reserve polysaccharide starch. It has some unique properties, which play an important role in breadmaking. On average, starch consists of 25% amylose and 75% amylopectin. Amylose is a largely linear molecule, consisting of α-(1,4)-linked D-glucopyranosyl units. Amylopectin is highly branched and is composed of chains of α-(1,4)-linked D-glucopyranosyl residues interlinked by α-(1,6)-glycosdic-bonds.

During milling around 8% of the starch granules are damaged. The degree of damage to the starch granules affects the starch properties, like water absorption and dough mixing properties. Damaged starch absorbs more water and is more susceptible to enzymic hydrolysis (Hoseney, 1994; Morrison et al., 1994) than native starch. The level of damage has an optimum and too much damaged starch is less suitable for wheat breadmaking. That is why durum wheat which has a hard endosperm structure, yields too much damaged starch after milling and is, therefore, less suitable for breadmaking than common wheat.

Starch granules can take up to 46% of their weight of water, when starch is heated up in the presence of water. This so called swelling of starch is a property of the amylopectin. In conventional cereal starches, amylose and lipids actively inhibit swelling (Tester & Morrison,

1990). In solution, amylose is a linear polymer, with tendency toward crystallization, which is known as amylose retrogradation. When the starch suspension is heated above a characteristic temperature (gelatinization temperature), a thermal disordering of crystalline structures in native starch granules occurs, including swelling of the granules and leaching of soluble polysaccharides from the granules.

The gelatinization temperature increases, if helical amylose-lipid complexes are present. Amylose forms a left-handed single helix and the hydrocarbon chain of the lipids, in particular of polar lipids, is situated in the central cavity. It was shown that the gelatinization temperature has also an influence on oven spring, bread volume and rate of crumb firming during staling (Soulaka & Morrison, 1985). The presence of polar lipids increases the gelatinization temperature resulting in a longer oven spring and a higher bread volume as well as in retarded bread staling.

During further heating, swelling of amylopectin and leaching of amylose continue and a suspension of amorphous starch granules and solubilized macromolecules is formed. This starch paste changes with time when it is cooled down. The starch polysaccharides reassociate to a more crystalline state. This process is called retrogradation (Atwell et al., 1988). The crumb structure becomes more firm and less elastic, combined with a loss of water. Bread staling is a complex phenomenon, but the most important factor is the water migration and transformations in the starch part. Amylopectin retrogradation mainly contributes to firming of the crumb, in particular, formation of double-helical structures and crystalline regions. The stale bread can be refreshed by heating to 50-60 °C because of melting of the crystalline amylopectin portion (Hug-Iten et al., 2003).

Addition of starch-degrading enzymes is a common strategy to retard firming of bread by altering the structure of starch and the soluble carbohydrate profile. Intermediately thermostable maltogenic amylase is one of the most effective anti-staling agents. It produces mainly α-maltose from starch. This enzyme has a maximum activity above the gelatinization temperature of wheat starch, but is inactivated during baking. Hug-Iten et al. (2003) suggested that the enzyme degrades the amylopectin side chains, thus hindering double helix and crystal formation and cross-linking between amylose and amylopectin. Further, the formation of crystalline amylose is enhanced that hinders rearrangements of the amylose phase and prevents structure collapse. Application of anti-staling agents should provide a balance

between a weakening of the starch structure, the formation of a stabilized starch network, and a reduction of the crystallization rate and structure collapse.

1.1.2.2 Non-starch polysaccharides

Non-starch polysaccharides is a generic term for arabinoxylans, β-glucan, cellulose and arabinogalactan-peptides (Goesaert et al., 2005). Wheat endosperm cell walls constitute up to 75% (dry matter weight) of non-starch polysaccharides of which arabinoxylans are the most prominent group (85%). Arabinoxylans are polymers of β-(1,4)-linked D-xylopyranosyl residues, substituted with monomeric α-L-arabinofuranoside. Ferulic acid can be coupled to arabinose by an ester linkage. Arabinoxylans form highly viscous solutions when dissolved in water and their water binding capacity is much higher than that of starch. 25-30% of the arabinoxylans in wheat flour endosperm is water extractable because it is loosely bound at the cell wall surface. Water-extractable arabinoxylans increase the viscosity of the free water phase. This stabilizes the gas cells and prolongs the oven spring resulting in higher bread volume (Gan et al., 1995). In contrast, water-unextractable arabinoxylans enhance gas cell coalescence and decrease gas retention, thus providing a poorer bread quality (Courtin & Delcour, 2002).

1.1.2.3 Proteins

Of all seeds in the plant kingdom, the wheat grain alone has the gluten proteins capable of forming the unique cohesive dough with viscoelastic properties when mixed with water, which is capable of retaining gas and setting in the oven during baking. These properties are essential prerequisites for the production of large specific volume and relatively uniform gas cells. According to their solubility, cereal proteins are divided into water-soluble albumins, salt soluble globulins, prolamins soluble in aqueous alcohols, and glutelins, which are only soluble in aqueous alcohols after reduction of disulfide bonds (Osborne, 1924). From the functional point of view, two groups of wheat proteins should be distinguished: the non-gluten proteins and the gluten proteins. Wheat gluten is defined as the insoluble residue which remains after washing out starch and soluble constituents from a dough. Gluten, consisting of gliadins and glutenins, has a major role in breadmaking (Goesaert et al., 2005). Gliadins and

glutenins are the major storage proteins of wheat. On the basis of the amino acid sequences, amino acid compositions, and molecular weights, gliadins can be classified into four different types: α-, γ-, ω5-, and ω1,2-gliadins (Shewry et al., 1986). Glutenins are polymeric proteins consisting of both high molecular weight glutenin subunits (HMW-GS) and low molecular weight glutenin subunits (LMW-GS). The glutenin subunits can be liberated from the polymers by reduction of disulfide bonds with reducing agents such as dithiothreitol. Nowadays, the protein composition of cereals can be analyzed by a modified Osborne fractionation followed by reversed-phase high-performance liquid chromatography (RP-HPLC). Using RP-HPLC, the proteins are separated according to their polarity (Wieser & Seilmeier, 1998; Wieser, 2000) with polar proteins eluting earlier than nonpolar ones.

The functions of gliadins and glutenins during dough preparation are divergent. Hydrated gliadins have little elasticity and are less cohesive than glutenins; they contribute mainly to the viscosity and extensibility of wheat dough. In contrast, hydrated glutenins are both cohesive and elastic and are responsible for dough strength and elasticity. A higher ratio of gliadins to glutenins results in a more viscous and softer dough. Common wheat has a ratio of gliadins to glutenins between 1.5 and 3.1 (Wieser & Koehler, 2009). An optimal ratio of gliadins to glutenins is important in the breadmaking process, because together they form gluten, a continuous protein network that gives wheat dough its unique viscoelasticity and gas-holding capacity after the mixing of flour with water. Due to the large size, glutenin polymers form fibrils and strands which provide resistance to deformation and elasticity to the dough (Belton, 1999). On the other hand, the monomeric gliadins are believed to act as plasticizers of the glutenin polymeric system. Without the gliadins, the glutenin interactions would form a very stiff gel which inhibits the gas cell expansion. The gluten quality is dependent on different factors. Beside the quantity, the composition of gluten is of greater importance. Differences in the glutenin subunits composition result in differences in the non-covalent bonds and disulfide bonds, which are responsible for the elasticity of gluten.

During dough mixing the gluten proteins are transformed into a continuous viscoelastic gluten protein network. The gluten development during dough mixing and energy input can be monitored by a Farinograph or a Mixolab instrument. The optimal mixing time is reached when the resistance of the dough has reached its maximum. At this point, an optimally developed gluten network is present, which provides high gas retention capacity. During further mixing, shorter and more irregular protein films are formed resulting in a lower gas retention capacity (called ‘over-mixing’). During dough fermentation and the initial stages of

baking, the gluten network is mainly responsible for retaining the carbon dioxide generated by yeast. During baking, the gluten network is further expanded by the oven spring before heat-induced denaturation of the proteins occurs. The typical open foam structure of baked bread is formed because of changes in the protein surfaces as well as the transformations of starch.

The functionality of gluten proteins can be influenced by oxidizing and reducing agents which impact the thiol-disulfide system of the glutenin molecules resulting in changes in rheological properties of dough. In particular, the formation of very large glutenin polymers (glutenin macropolymer, GMP) by inter-chain disulfide bonds is important for dough strength and loaf volume (Weegels et al., 1996). Chemical oxidizing agents, like potassium iodate and potassium bromate, oxidize free SH groups in dough and increase the strength of gluten. In contrast to ascorbic acid, both halogenates do not require molecular oxygen to exert their improving effect. Nowadays, the use of bromate has been largely banned from the baking industry because of toxicological studies. Ascorbic acid is the most accepted additive and is widely used, mainly in countries where bromate is not permitted. Actually, L-threo ascorbic acid is not the effective reagent, but its oxidation product dehydro-L-ascorbic acid. Beside chemical oxidation mediated by iron and copper ions, L-threo-ascorbic acid is also oxidized enzymatically in the presence of atmospheric oxygen. The improving effect of dehydro-L-ascorbic acid is based on a rapid removal of endogenous glutathione, which otherwise would cause dough weakening by sulfhydryl/disulfide interchange with gluten proteins (Grosch & Wieser, 1999). More and more exogenous enzymes are used to affect the endogenous redox system of wheat flour. Typical examples are glucose oxidase, peroxidases, and lipoxygenase. By addition of transglutaminase to wheat dough, the properties of the bread can be positively influenced similar to the effects of oxidants. Transglutaminase catalyzes the acyl-transfer reaction between the side chains of L-lysine and L-glutamine, thereby forming inter-chain isopeptide cross-links. Unlike transglutaminases, peptidases catalyze the irreversible partial hydrolysis of gluten proteins leading to weaker doughs. The peptide bonds and the initial rheological properties cannot be restored, which is in contrast to reducing agents such as cysteine or metabisulfite. Peptidases can be used to shorten the mixing time, to decrease the dough consistency, to control bread texture, and to improve flavor.

In addition to salt and alcohol-soluble proteins, there is a small proportion of proteins that require detergents (Triton X114) for solubilisation. In particular puroindolines (PINs) strongly affect the breadmaking quality of wheat flour. The two major isoforms, PIN-a and PIN-b are found in the starchy endosperm and are attributed to soft texture. Durum wheat, the hardest of

all wheat species, has no PINs. One factor for endosperm hardness is the adhesion between the starch granules and the protein matrix of the endosperm. The protein complex was found to be associated with the starch granule surface in soft milling wheat (Greenwell & Schofield, 1986), whose major components are PINs. These proteins produce a less sticky surface of starch granules, thereby reducing the adhesion with the endosperm matrix. PINs also have a unique tryptophan-rich domain, which has a lipid binding capacity. PINs are essential to the foaming properties of dough liquor, and a close relationship was found between the fine crumb grain provided by reconstituted flours with PINs and the fine structure of the corresponding dough liquor foams (Dubreil et al., 1998). These highly surface-active proteins still show excellent foam stabilizing properties even in the presence of lipids, which have generally detrimental effects on foam quality. Pauly et al. (2014) suggested that both endogenous PINs and lipids impact crumb structure by forming more surface-active lipid-PIN complexes.

1.1.3 Wheat flour lipids

Although they are present in wheat in lower levels than starch and protein, lipids exhibit important functional properties in breadmaking. The total lipid content in wheat grain is about 3-4 weight% and in wheat flour usually 1-2.5% depending on genetic and environmental factors, as well as milling conditions (Chung, 1986). 30-45% of wheat lipids are located in the germ, 25-29% in the aleurone layer, and 35-45% in the endosperm (Hargin & Morrison, 1980). Germ and aleurone lipids are mainly non-polar (77-85% and 72-83%, respectively). The composition of wheat flour lipids, mainly endosperm lipids, is different from that of germ and aleurone lipids. Wheat flour lipids provide a substantial source of polar lipids, with galactolipids (monogalactosyl diglycerides [MGDG] and digalactosyl diglycerides [DGDG]) and phospholipids (N-acyl-phosphatidyl ethanolamine [NAPE], phosphatidyl ethanolamine [PE], phosphatidyl choline [PC], lysophosphatidyl choline [LPC]) being predominant (Hargin & Morrison, 1980; Finnie et al., 2009).

Wheat flour lipids are divided in two major groups based on their apparent functionality. Two-thirds form the non-starch lipids and one third the starch lipids. Starch lipids are strongly associated with the starch granule matrix. Starch lipids are mainly lyso-phospholipids and bound as inclusion complexes with amylose in the starch. Starch lipids are only extractable at

temperatures above the starch gelatinization temperature using a polar solvent (e.g. water-saturated *n*-butanol (WSB)) at 90 °C (Morrison et al., 1975). The non-starch lipids are extractable at room temperature and can be further subdivided into free and bound lipids according to their different solubility. Free lipids are extractable with non-polar solvents, e.g. petroleum ether or hexane. Bound lipids are associated with wheat proteins and can be extracted with more polar solvents, e.g. WSB or 2-propanol:water (90:10) (Hoseney et al., 1969; Finnie et al., 2009). Free and bound lipid extracts build the total non-starch lipid fraction. The lipid composition is dependent on solvent polarity, extraction temperature and the particle size of the sample. Further, the separation of free and bound lipids does not provide a distinct division between non-polar and polar lipids (Chung et al., 1980). However, free lipids generally contain mainly the non-polar fraction, tri-, di-, monoacylglycerides and sterol esters, and the bound lipids contain mainly the polar galactolipids and phospholipids.

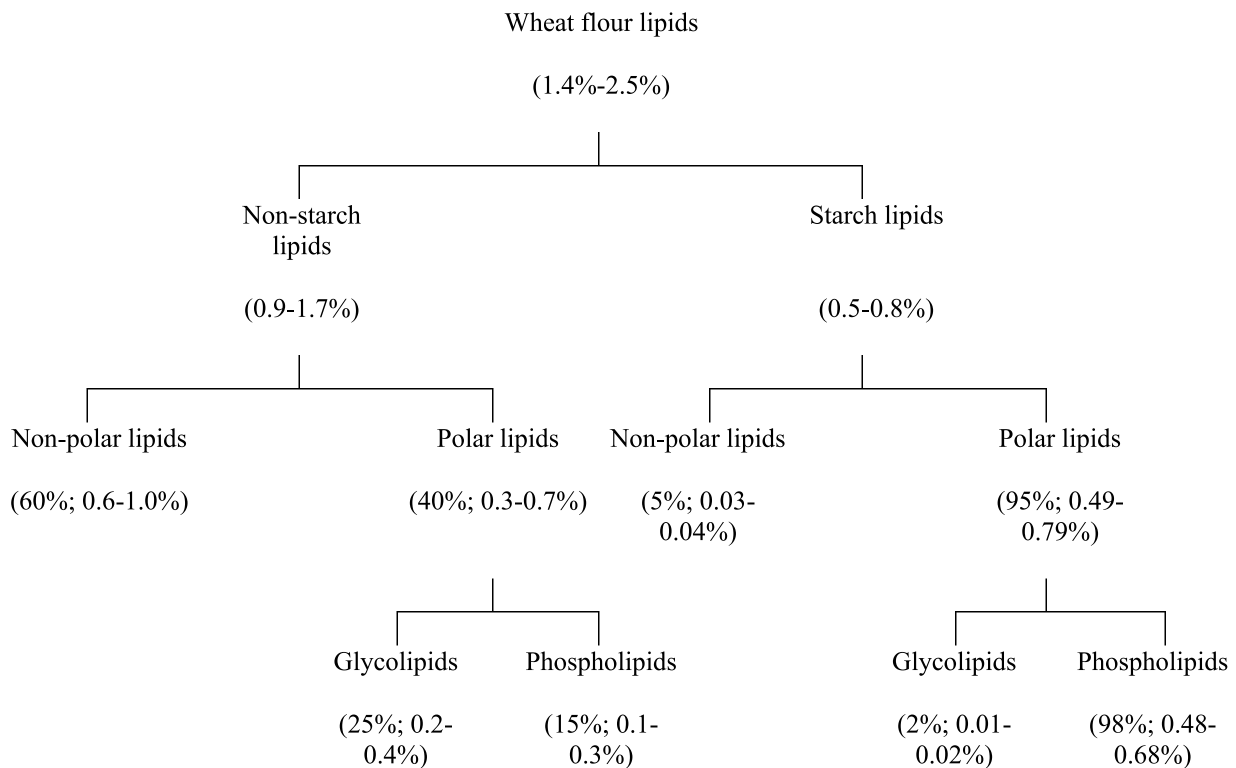

Figure 1.1: Wheat flour lipids: Distribution of non-starch and starch lipids (Source: Morrison et al., 1975).

About 70% of the free lipids are non-polar containing triglycerides as a major lipid class. With the bound lipids it is the opposite way round. The polar lipids, the major building blocks of every cell membrane, are a mixture of glycolipids and phospholipids. Bound polar lipids are rich in phospholipids with LPC as a major component. Free polar lipids of wheat flour are

enriched in glycolipids whereas bound polar lipids are slightly enriched in phospholipids (Pomeranz & Chung, 1978). The total lipid composition of a high-grade spring and high-grade winter wheat flour (dry base) as found by Morrison et al. in 1975 is shown in Table 1.1.

Table 1.1: Lipid composition of a high-grade spring wheat flour (HGS) and a high-grade winter wheat flour (HGW) according to Morrison et al. (1975).

	HGS wheat flour		HGW wheat flour	
	Non-starch[1]	Starch[2]	Non-starch[1]	Starch[2]
Lipid	mg/100 g		mg/100 g	
Sterol esters (SE)	72	18	43	10
Triglycerides (TG)	674	35	909	10
Diglycerides (DG)	86	6	67	3
Free fatty acids (FFA)	110	19	64	15
Acylated monogalactosyl diglycerides and monoglycerides (MG)	66	7	53	3
Acylated sterol glycosides (ASG)	71	6	18	3
Monogalactosyl diglycerides (MGDG)	87	6	115	2
Monogalactosyl monoglycerides (MGMG)	23	7	17	5
Digalactosyl diglycerides (DGDG)	214	12	322	3
Digalactosyl monoglycerides (DGMG)	58	25	52	9
N-Acyl phosphatidyl ethanolamine (NAPE)	72	/	95	/
N-Acyl lysophosphatidyl ethanolamine (NALPE)	34	/	33	/
Phosphatidyl ethanolamine (PE) and phosphatidyl glycerol (PG)	13	6	19	/
Lysophosphatidyl choline ethanolamine (LPE) and lysophosphatidyl glycerol (LPG)	10	59	12	127
Phosphatidyl choline (PC)	66	38	96	/
Lysophosphatidyl choline (LPC)	36	657	29	615
Phosphatidyl serine (PS), lysophosphatidyl serine (LPS), phosphatidyl myo-inositol (PI) and lysophosphatidyl myo-inositol (LPI)	11	24	9	30
Total non-polar lipids	1079	91	1154	44
Total glycolipids	382	50	506	19
Total phospholipids	242	784	293	753
Total lipids	1703	925	1953	816

[1] Non-starch lipids extracted with WSB at 20 °C
[2] Starch lipids extracted with WSB at 90 °C

The fatty acid composition of wheat flour lipids is dominated by linoleic acid (C18:2) with lower amounts of palmitic (C16:0) and oleic acid (18:1) (Hoseney, 1994).

Despite their low levels endogenously present in wheat flour, lipids were found to significantly affect the baking performance of wheat flour. A detailed overview can be found in Chung et al. (2009) and Pareyt et al. (2011).

It was established that the improvement in bread volume was mainly due to the polar lipids. Especially the glycolipids are better improvers than the phospholipids. During the first stages of mixing, the bound lipid level increases at the expense of the free lipid level. Around two-thirds of the free lipids, some non-polar and almost all polar lipids, become bound in dough when gluten forms. Starch lipids do not play a role during dough mixing because they are tightly bound in the starch granules and have no significant effect on dough properties. McCann et al. (2009) suggested that galactolipids interact with the glutenins through hydrophobic and hydrogen interactions, while phospholipids would interact with the gliadins or lipid binding proteins of gluten. The binding of free lipids to gluten proteins may stabilize the gas cells during breadmaking through alignment at the interface of gas cells. Sroan and MacRitchie (2009) showed that endogenous lipids do not have any direct influence on the rheological properties of dough, indicating the importance of flour lipids as surface-active components either stabilizing or destabilizing the gas cell structure of dough. Free and bound non-starch lipids contribute to the bread volume (McCormack et al., 1991). It was shown that the endogenous lipid content of the wheat dough does not affect dough volume during proofing but during the oven spring (Klingler, 2010; Gerits et al., 2015a). The improving effect is much greater for lipids than for proteins in regard to their concentration (MacRitchie, 1981). McCormack et al. (1991) came to the conclusion that lipids only have a minor contribution to the loaf volume differences between cultivars. In some cases, the strong influence of protein content might mask the positive effect of free polar lipids in bread volume. Reconstitution baking tests with increased polar lipid fraction at constant total lipid content showed a linear increase of the loaf volume (McCormack et al., 1991).

MacRitchie and Gras (1973) found that polar lipids appear to have stabilizing effects on gas cells above a critical concentration. Adding the extracted polar lipid fraction back to flour defatted with chloroform, the minimum in bread volume was reached at a low level of added lipids. After increasing the lipid content, the volume increase was stronger for the polar lipid fraction compared to the total lipid fraction (Figure 1.2). The whole lipid fraction added back to defatted flour decreased the bread volume to a minimum at a lipid content intermediate between the defatted and untreated flour. The bread volume increased to a value comparable to the volume of bread from untreated flour after adding back the original amount of extracted

lipids to defatted flour. A further increase in the level of total extracted lipids brought a further increase in bread volume. The non-polar lipid fraction produced a progressive decline in loaf volume, whereas mainly the unsaturated FFA reduce bread loaf volume and the saturated FFA do not (De Stefanis & Ponte, 1976; Sroan & MacRitchie, 2009). As doughs prepared from defatted flours can expand, it must be assumed that surface-active lipids are not necessarily essential for gas retention and that the proteins dissolved in the dough aqueous phase can take over the functions of lipids in defatted flour (MacRitchie & Gras, 1973; Gan et al., 1995). However, there is no doubt that polar lipids improve bread loaf volume and crumb grain, whereas non-polar lipids have the opposite effects.

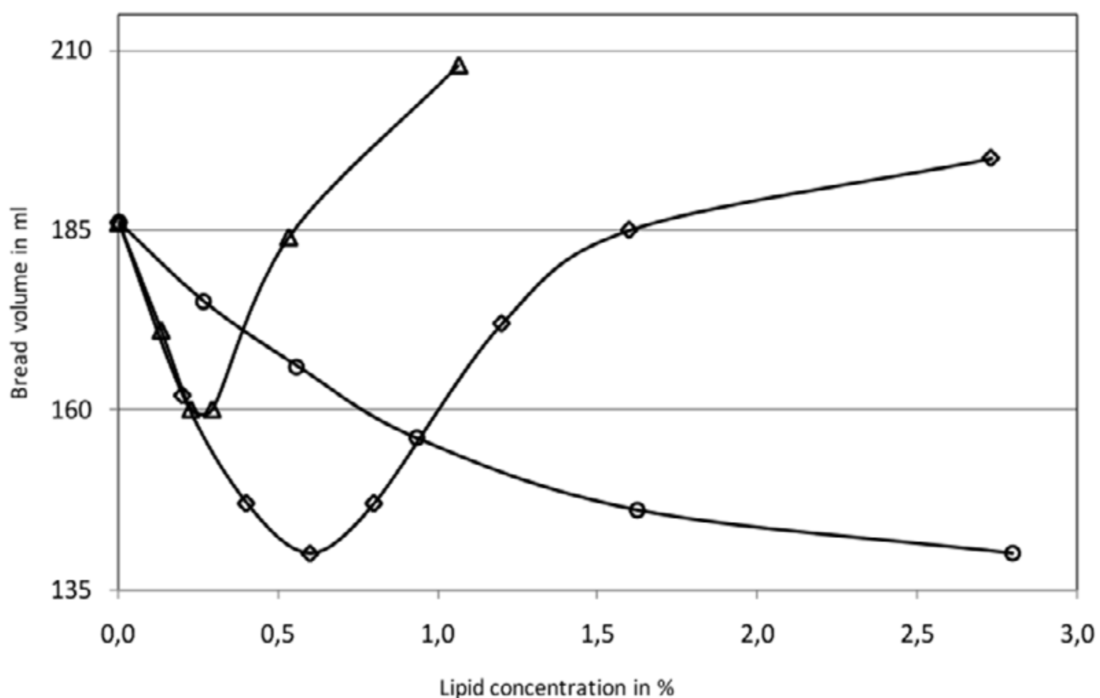

Figure 1.2: Bread volume (ml) as affected by the lipid concentration (%) of wheat flour when extracted lipids were added back to defatted flour. ◊ = extracted total lipids; Δ = extracted polar lipids; ○ = extracted non-polar lipids (data taken from MacRitchie & Gras, 1973).

Wheat lipids form lipid monolayers or single lamellar phase systems at the gas-liquid interface. The orientation is based on their amphiphilic character and their structure, whereas gaseous, expanded liquid and condensed liquid films are possible. In gaseous films the molecules lie nearly flat on the water surface and the lipid molecules do not interact with each other. Condensed monolayers of surface-active molecules are closer packed with the polar head groups oriented toward water and non-polar hydrocarbon chains toward air. Expanded

monolayers are characterized by loose packing of surface-active molecules with a much higher area per molecule. Condensed monolayers are the most stable barrier against coalescence of gas cells because they are more elastic and relatively incompressible, providing resistance to collapse of the liquid lamellae (MacRitchie, 1976; Sroan & MacRitchie, 2009). In defatted flour, gas cells are only stabilized by surface-active proteins resulting in a relatively high loaf volume and a fine and uniform crumb structure. Proteins provide a highly condensed state of a single component and do not desorb from the gas-liquid interface easily. Addition of lipids or lipid fractions back to defatted flour causes a destabilization of protein films by formation of mixed protein/lipid monolayers. Salt et al. (2006) showed that pure protein interfaces are highly surface-elastic and more stable compared to mixed protein-lipid interfaces. Further addition of lipids, especially polar lipids results in formation of lipid-dominated films (Figure 1.3) and restores the properties of the initial protein films.

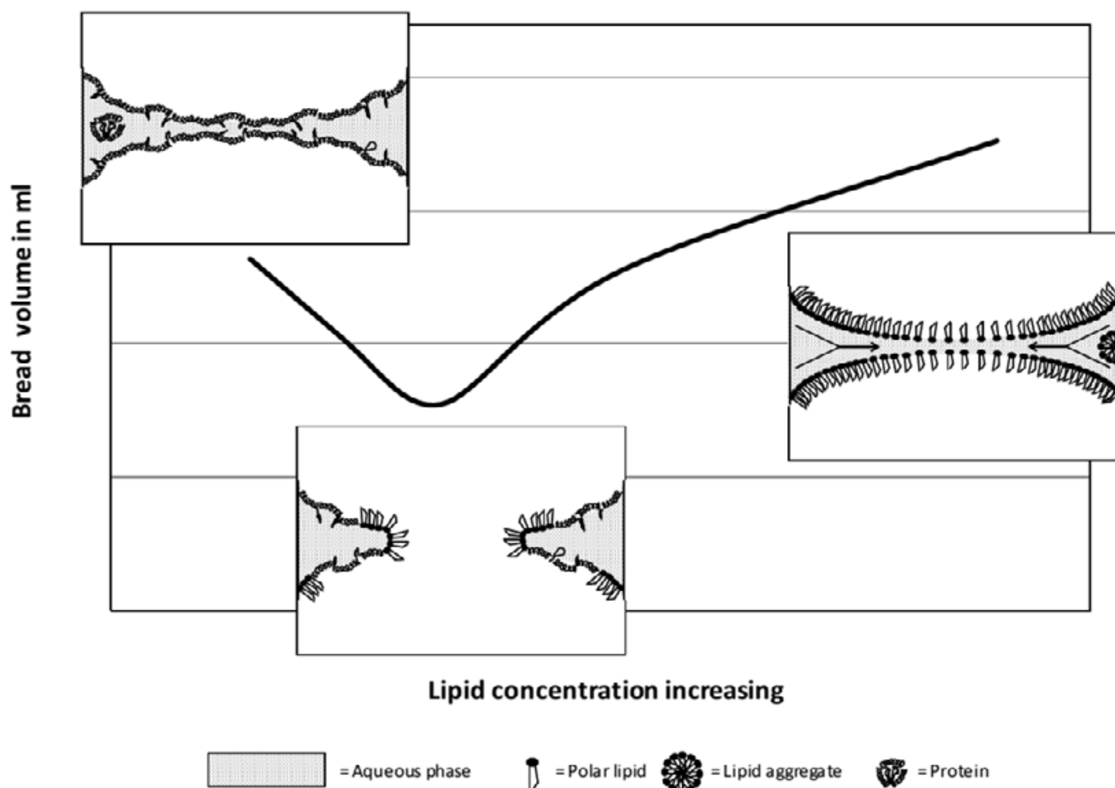

Figure 1.3: Liquid film stabilzation in wheat dough and bread volume as affected by the concentration of polar lipids as proposed by Gan et al. (1995)

Sroan & MacRitchie (2009) showed that non-polar lipids, linoleic and myristic acid form expanded monolayers, whereas polar lipids and palmitic acid form condensed monolayers at the air-water interface. The latter fatty acids had either no effect or a positive effect on loaf

volume. Double bounds in fatty acids, especially in the *cis* conformation, prevent the close packing of monolayers, resulting in an expanded orientation (Gaines, 1966). Polar lipids promote the lamellar crystalline lipid-water phase (Figure 1.4), which favors the formation of condensed monolayers. The lamellar phase is favored by lipids with saturated fatty acid moieties. Other mesophases are the hexagonal II and the hexagonal I lipid – water phase. Hexagonal II phases are favored by relatively nonpolar lipids with a low hydrophilic lipophilic balance (HLB) value and hexagonal I phases by polar lipids with high HLB values. Gerits et al. (2014) speculated that the stabilization of gas cells is based on lipids favoring the lamellar phase which forms condensed monolayers. Lipids favoring the hexagonal phase I may emulsify the deleterious non-polar lipids by formation of micelles and thereby preventing their absorption into the interface. Altogether, the differences in the gas-cell stabilizing properties of the various wheat flour lipid classes are due to differences in the ability to form monolayers at the gas-liquid interface (Sroan & MacRitchie, 2009).

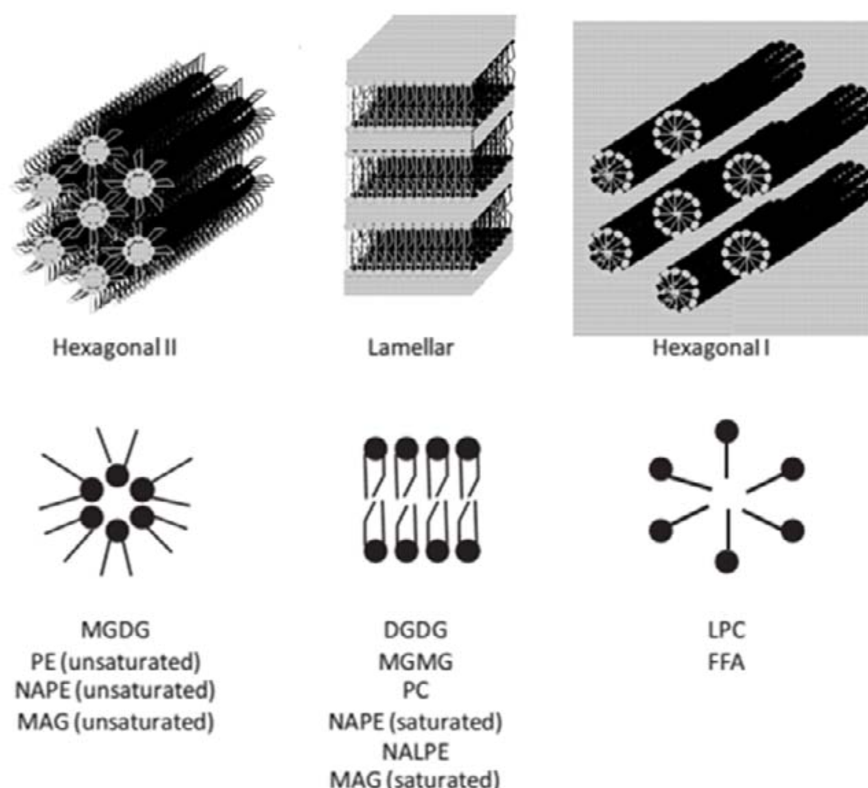

Figure 1.4: Mesomorphy of different polar lipid classes present in wheat based on Gerits et al. (2014b) and Selmair (2010).

Glycolipids with one carbohydrate moiety, like MGDG, exhibit non-bilayer forming properties, but glycolipids with two sugar groups (e.g. DGDG) always form bilayers (Hoelzl

& Doermann, 2007). In wheat grains, non-bilayer lipids are always present either in the glycolipid fraction (MGDG) or in the phospholipid fraction (NAPE). Selmair (2010) confirmed the negative influence of non-polar lipids and NAPE by micro-baking tests with different glycolipid classes and samples enriched with glycolipids from lecithin. All glycolipid classes tested had a highly positive effect on the bread volume, with ASG, cerebrosides (CBR) and DGDG showing a better baking performance than the classical surfactants like DATEM and stearoyl-2-lactylate (SSL). The experiments showed that the hydrophilic and lyophilic parts in the molecular structure of each lipid class are important for its baking potential. Glycoglycerolipids with one carbohydrate moiety and a monoacyl compound possess a far better hydrophilic lipophilic ratio in comparison to the diacyl compound (Selmair & Koehler, 2008).

Among the polar lipids, the glycolipids were a better loaf volume improver than the phospholipids (Chung et al., 1980). From the phospholipids, PI was reported to increase bread volume when added in low concentrations of 0.02–0.1% (Helmerich & Koehler, 2005). High proportions of PI and PE decreased the loaf volume. Furthermore, it was shown that LPC is more active than PC. With addition of small amounts of baking-active glycolipids to phospholipids (commercial lecithin) a significantly better functional effect was achieved. The best synergistic baking performance of glycolipid/phospholipid mixtures was established with a ratio of 1/6 (w/w) (Selmair, 2010).

In summary, both the composition and structure of wheat flour polar lipids influence the stabilization of gas cells and finally the quality of the bread. Selmair & Koehler (2009) proposed the categorization of lipids in modes of action with a direct or indirect stabilization effect. Possible modes of action are the direct effect of lipids on the liquid film lamellae and gas cell interfaces through direct adsorption and an indirect effect on the interface based on synergistic effects between lipids and the different constituents of the dough. The mesomorphy type and the monolayer type formed at the interface are responsible for the surface-activity of the lipids. The positive direct mode of action is based on formation of condensed monolayers and prevention of gas cell coalescence (Figure 1.5A). The positive indirect mode of action is mainly based on lipid-lipid or lipid-protein interactions. Synergistic effects between lipid classes could occur because monolayers consisting of a suitable mixture of lipid classes with different mesomorphy types are similarly stable than monolayers of lipids of the lamellar type (complementary effect). Mixed micelles with different lipid classes reduce the energy barrier to adsorb to the interface and therefore increase the availability of

surface-active lipids at the interface (Figure 1.5B). Furthermore, non-polar lipids can be emulsified by polar lipids and the adsorption of the deleterious non-polar lipids at the interface is prevented. By lipid-protein interactions, lipids can from associations with membrane proteins and lipid binding proteins, especially puroindolines, showing increased surface activity (Dubreil et al., 1997). Moreover, additional surface-active material at the interface can be provided by solubilisation of suitable gluten proteins by polar lipids.

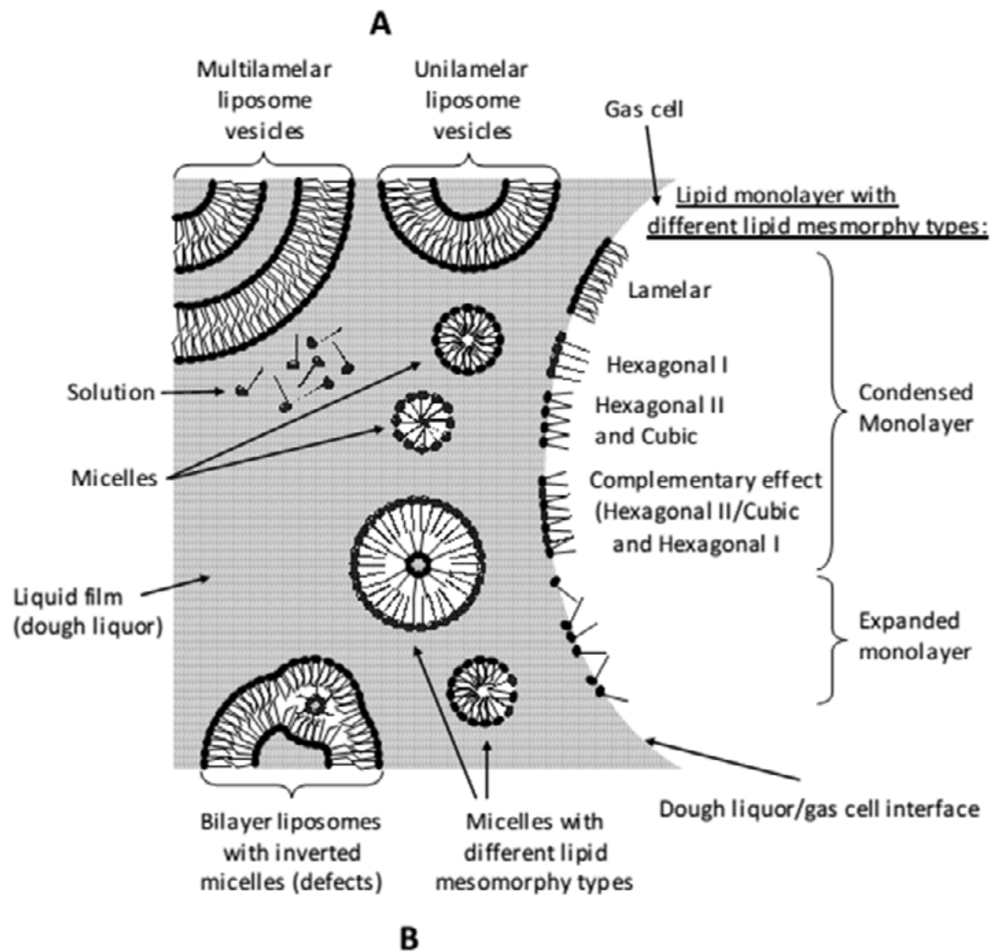

Figure1.5: Modes of action of lipids present in dough liquor and gas-liquid interfaces proposed by Selmair (2010): Direct (A) and indirect (B) stabilizing effect of lipids on the dough liquor–gas cell interface through a lipid monolayer.

1.2 Surfactants

Surface-active agents, or so-called surfactants, are molecules with amphiphilic properties, i.e. they contain both hydrophilic and hydrophobic parts. Surfactants are used as additives in the baking industry since 1930 to compensate variations of the flour quality and to ensure

consistent product quality. All surfactants are polar lipids, which are naturally occurring in small amounts in wheat or are synthetically related.

Emulsifiers, or surfactants, are surface-active molecules that adsorb to the interface of two or more immiscible liquids, thereby forming an interfacial film and so help to stabilize the emulsion by lowering the interfacial tension between the water and other liquids. This change in the interfacial tension causes the formation of a physical barrier around each droplet to stop the coalescence and the formation of two separate large phases (Selmair, 2010). In breadmaking three colloidal systems, emulsion (liquid / liquid), gel (solid / liquid) and foam (liquid / gas), occur. Surface-active molecules are of great importance to stabilize the various interfaces resulting in improved dough handling, higher bread volume, better crumb structure and softness and also in the retardation of bread staling (Stampfli & Nersten, 1995; Gray & Bemiller, 2003).

1.2.1 Surface-active molecules and interfaces

Due to their amphiphilic character, surfactants always orientate themselves at gas/liquid or polar/non–polar interfaces. The surface energy between the water and gas phase or the interfacial energy between oil and water is reduced by surface-active molecules.

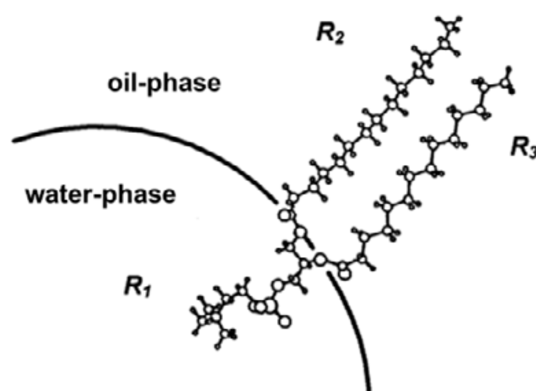

Figure 1.6: Orientation of the polar lipid 1,2-dimyristoyl-glycero-3-phosphatidylcholine at the oil/water–interface. R_1: Glycero-phosphatidylcholine; R_2, R_3: myristic acid.

In wheat flour, polar lipids and proteins are the surface-active molecules. When wheat dough is fermented, the colloidal structure is transformed from a dispersion of gas cells into a foam system. The foam consists of gas cells encapsulated by lamellae of liquid. Surfactants

improve the stability of gas cells in dough, particularly during the later stage of proofing, when the gas cells are thought to be stabilized only by thin liquid films (Gan et al., 1995). If sufficient amounts of emulsifiers are available to reach the gas cell interface in dough, it is likely that they will expel the proteins from the interface and will be the only constituents left in the gas cell film (Keller et al., 1997). Therefore, they are thought to shift the stability regime from a less stable mixed lipid-protein system over to a surfactant-dominated system, as seen with higher concentration of polar lipids (see Figure 1.3). At the one hand, surfactants in dough reinforce the gluten network through interaction with gluten proteins and on the other hand they support the endogenous flour lipids in their foam-stabilizing function, through formation of ordered lamellar mesophases in water (Schuster, 1984; Schuster & Adams, 1984). In addition, anti-staling effects have been described for some surfactants, in particular monoglycerides (MG) (Schuster, 1984; Schuster & Adams, 1984). Table 1.2 lists the positive effects of surfactants during the baking process, starting from the mixing, fermentation and baking, the direct effects, and storage of the baked good, the indirect effect.

Table 1.2: Direct and indirect effects of surfactants in breadmaking according to Selmair (2010)

Process step	Effect	Cause for effect	Mode of action
Direct effect			
Mixing	Decrease of mixing time and speed; Reduction of shortening levels; Improved dough handling (machinability)	Improved rate of hydration and water absorption; Better distribution of shortening	
Fermentation	Improved bread volume; Shorter fermentation; Improved shock-tolerance	Improved gas retention ; Improved dough stability; Stabilization of distributed phases; Improved rheological properties of dough, dough liquor, and gluten; Improved gas cell stability	Interactions of surfactants with • Water • Proteins • Carbohydrates • Lipids
Baking	Improved bread volume; Improved crumb-softness; Better texture; Better crumb grain; Better uniformity; Decrease of water loss		
Indirect effect			
Storage	Retarded bread staling; Longer shelf live	Retarded retrogardation of starch; Anti-staling effect	Formation of inclusion complexes between surfactants and starch

1.2.1 Classification

Surfactants can be classified according to various aspects, such as their different hydrophilic or lipophilic groups, their solubility properties, their HLB values, the possibility for ionization (non-ionic or ionic surfactants), their crystal structure or their behavior when in contact with water.

The most important classification method for surfactants is according to their HLB value. All surfactants, endogenous or synthetic, consist of molecular structures with hydrophilic and hydrophobic structural elements (Selmair & Koehler, 2008). Griffin (1949, 1954) introduced the HLB-value, which expresses the specific hydrophilic/lipophilic balance of the structural compounds, as index ranging from 0 to 20 that conveyed the affinity of a non-ionic surfactant to oil or water. These empirical HLB values were determined analytically. The individual HLB_G values of a non-ionic surfactant are calculated on the basis of the ratio between the molecular weight of the hydrophilic part of the molecule (M_h) and the molecular weight of the entire molecule (M):

$$HLB_G = 20\ (M_h/M)$$

Lipophilic surfactants were assigned a low HLB value and hydrophilic a high HLB value. The HLB value of a surfactant may be used as a guide to its most appropriate application.

However, for ionic surfactants a system proposed by Davies (1957), in which numerical values were assigned to the individual functional groups of surfactants, has to be used. Subsequently, the HLB scale was increased up to a HLB value of 40 to include also the ionic substances:

$$HLB_D = \Sigma\ (\text{hydrophilic group numbers}) - \Sigma\ (\text{lipophilic group numbers}) + 7$$

1.2.2 Commercial surfactants for the baking industry

1.2.2.1 Mono- and diglycerides

The most important surfactants are the synthetic MG and diglycerides (DG) with about 70% of the word production of food surfactants. They had their major breakthrough in the 1930s when they were used on an industrial scale in the margarine industry (Krog et al., 2004).

Today there are approximately 20 different types of MG and DG and their derivatives used in food products. The biggest application field today is in bakery products, in particular in bread, sponge cake, and cake production. The most important feature of MG in bakery applications is their capability to form inclusion complexes with amylose and amylopectin and, hence, prolonging the freshness of the bread structure. Further, MG can also act as bread crumb softeners. Strandine et al. (1951) suggested that small particles of MG cover the surface of starch granules and reduce the swelling of starch and the release of water-soluble components.

The synthesis of MG and DG can be accomplished either by interesterification between triglycerides and glycerol ("glycerinolysis"), or by direct esterification of glycerol and a fatty acid, which takes place at 200 °C and alkaline catalysis. To separate the MG from the DG, triglycerides (TG) and glycerol in the resulting reaction mixtures, a thin film distillation under high vacuum at temperatures between 140-170 °C has to be used. This separation technique results in the following composition of a MG distillate: 95% MG, 3-4% DG, 0.5-1% free glycerol and 0.5.-1% free fatty acids (FFA) (Krog et al., 2004). MG are soluble in oil and are classified with low HLB values from 3 to 6.

1.2.2.2 Diacetyl tartaric acid esters of monoglycerides

MG esterified with mono- and diacetyltartaric acid (DATEM) are very popular anionic oil-in-water emulsifiers that are used throughout the world as improvers in breadmaking. DATEM is produced by the reaction of mono- and diacetyltartaric acid anhydride with MG or mixtures of MG and DG. It improves the bread texture and volume, slows down bread staling, increases the dough tolerance and positively affects dough properties during proofing and baking (Mettler et al., 1991; Koehler & Grosch, 1999, Koehler, 2001a, 2001b, 2001c). The effect of DATEM depends on its composition and on the baking performance of the flour. Koehler (2001a, 2001b, 2001c) provided information about the influence of the fatty acid on the baking performance and the activity of individual components of DATEM. The three main active components of DATEM differ in the structure at the secondary hydroxyl group of glycerol, which may be free (P5-8-1) or esteried with acetic acid (P3-10-1) or diacetyltartaric acid (P5-12-1) (Figure 1.7). From these results, the author concluded that the anionic emulsifier DATEM neutralizes the positive charge of the gluten proteins resulting in improved aggregation of gluten. Increased dough strength results in higher volume and shock resistance. At the dough stage, the DATEM component with two carboxyl groups is most

active. The other two DATEM components with only one carboxyl group are most important during baking for gas holding at higher temperatures, when low amounts of free water are present. Gerits et al. (2015) showed that DATEM neither extended the oven rise time during baking nor affected starch gelatinization. The increased height at the end of the dough rise was due to an increased dough expansion and later gas cell opening caused by increased gas cell stability.

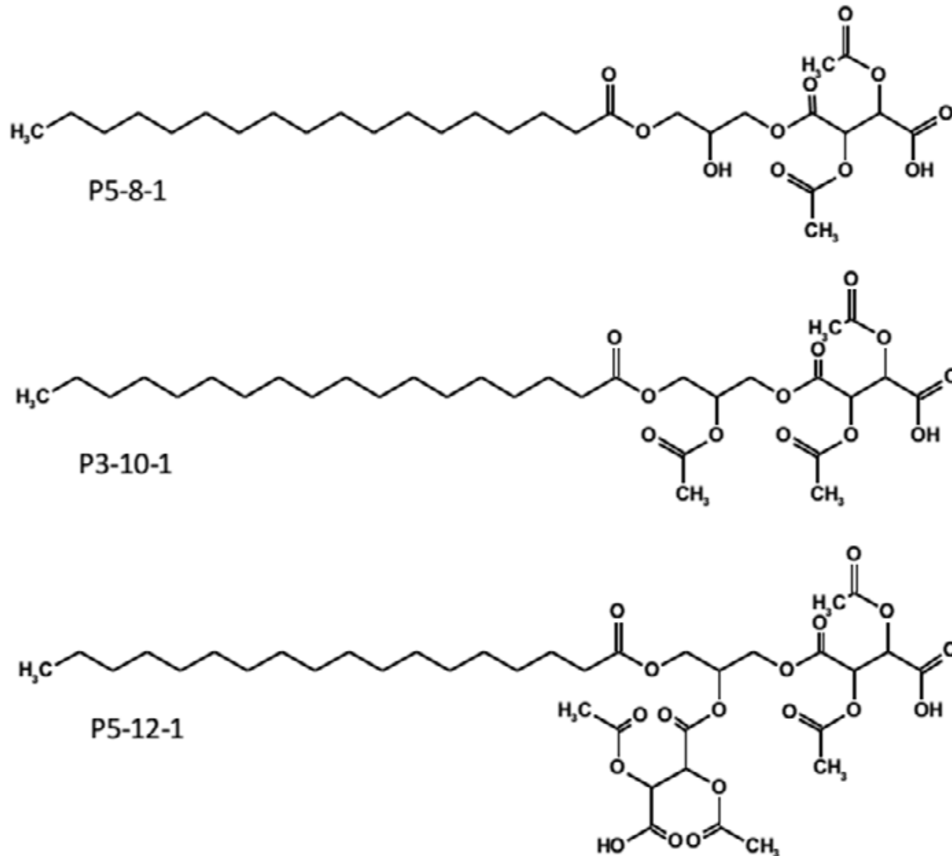

Figure 1.7: Main functionally effective components of DATEM as identified by Koehler (2001a, 2001b).

1.2.2.3. Stearoyl-2-lactylate

Beside MG/DG and DATEM, SSL is the third most important surfactant. Nowadays, the sodium salt is generally used in the majority of applications because of its better solubility in water as compared to calcium stearoyl lactylate (CSL).

SSL, as an anionic surfactant, can not only form stable emulsions but also strong complexes with both starch and protein. The success of SSL in breadmaking is based on the dual functionality of dough strengthening and crumb softening.

The dough strengthening effect of SSL has been attributed to its electrostatic interactions with gluten proteins during dough mixing, which may cause gluten aggregation and increase dough strength (Gómez et al., 2004). The crumb softening effect of SSL is due to the formation of inclusion complexes with starch, which retards bread staling.

De Stefanis et al. (1976) found that SSL is associated with the protein fraction from the beginning of dough mixing until the early stages of oven spring, when it then slowly transfers to the starch fraction and forms complexes. It was hypothesized that SSL preferably interacts with or binds to gliadins, limiting their participation in cross-linking reactions with glutenins (van Steertegem et al., 2013). Also, during baking, part of the SSL complexed with amylose. In contrast, DATEM remains bound to the protein fraction through the entire baking process and therefore does not interact substantially with the starch during breadmaking. This explains the poor bread crumb softening by DATEM in comparison to SSL and maybe also that DATEM yields more dough strength and volume than SSL.

1.2.3 Interaction between surfactants and wheat dough components

The main carbohydrate of wheat flour is starch, which is one of the important interacting partners of polar lipids, including also the surfactants.

Starch is present as solid granules with a high degree of organization. Together with dough liquor, starch forms a continuous phase in dough. Starch and the surfactant form a complex on the surface of the granules or as an inclusion. These complexes are dependent on temperature, concentration, physical structure, and geometric parameters. For a successful formation of an inclusion complex, the chain length and saturation of the fatty acid moieties is of major importance. The lipophilic interior of the starch helix is able to host the lipophilic fatty acid chains, resulting in a stable starch-surfactant complex. The surface of starch granules is hydrophilic. Hence, surfactants can interact with the surface of the starch granules with their hydrophilic head groups. This results in a hydrophobic coat, formed by the acyl chains, and hence to an agglomeration of the starch molecules. A further layer of surfactants can reverse

this effect, forming a hydrophilic surface once again. The effects caused by this interaction are reduced water absorption and swelling as well as an increase of the gelatinization temperature and viscosity of wheat starch (Krog et al., 2004). An important effect caused by the inclusion complex of amylose with the surfactant is a softer bread crumb and slower bread staling caused through slower recrystallization of the starch components. Saturated MG are the best anti-staling agents because the linear acyl chains fit better into the helix than unsaturated ones. Further, surfactants can build complexes with the short helical side chains of amylopectin, also resulting in slower recrystallization of amylopectin.

According to Greene (1975), the functionality of dough conditioners is primarily based on them binding to gluten proteins and hence increasing the aggregation of gluten proteins during mixing. With anionic surfactants, this is caused through reduction of the positive charge, hence reducing the electrostatic repulsion of the gluten molecules and leading to an increase in aggregation. Stauffer (1990) described the formation of ionic interactions between the gluten particles and anionic surfactants. The aggregation of gluten proteins is supported by hydrophobic interactions of non-polar ligands. The direct results of these interactions on the dough are an increased gluten strength with higher mixing tolerance of the dough and higher volume of the baked product. Another important effect is the improved shock tolerance of the dough after proofing.

Surfactants interact with non-polar lipids, in the presence and absence of water. Non-polar lipids are generally insoluble in water, forming micellar solutions when in contact with water. With surfactants and water present, the non-polar lipids micellar solution is stabilized forming an emulsion (Selmair, 2010). A synergistic effect between surfactant and endogenous polar lipids is possible. The additional source of surface-active material could promote the formation of lamellar mesophases resulting in increased gas cell stabilization.

Other authors postulated that DATEM competes with the endogenous lipids for interaction with gluten proteins (Gerits et al., 2015a).

1.3 Lipases for baking applications

1.3.1 Lipases

Lipases (EC 3.1.1.3.) are enzymes belonging to the hydrolase class (enzyme class 3). They hydrolyze lipids such as glycerides or cholesterol esters and release free fatty acids (lipolysis) and different products devoid of fatty acids. Lipases play an important physiological role in humans because they enable the digestion of dietary lipids (pancreatic lipase). Lipases and esterases differ in their substrate specificity. Lipases prefer lipophilic, water-insoluble substrates as well as triglycerides with short-chain fatty acids with limited water-solubility. They are active on the water/lipid interface. Esterases only hydrolyze short-chain, water-soluble substrates, like triacetin. In the absence of water, lipases possess the ability to carry out the reverse reaction, leading to esterification, alcoholysis and acidolysis.

A model for pancreatic lipases has been suggested to account for the enzyme's activity on the oil/water interface. The 'hydrophobic head' of the lipase is located at the enzyme surface near to the reactive site, but separate from it. The 'hydrophobic head' binds to the oil droplet by hydrophobic interactions and the resulting orientation of the lipase at the interface is further stabilized by a hydrophilic tail consisting of polar amino acid and carbohydrate residues (Figure 1.8). The active site aligns with and binds to the substrate molecule. The finer emulsified the oil droplets, the larger the oil/water interface and, therefore, the higher the lipase activity.

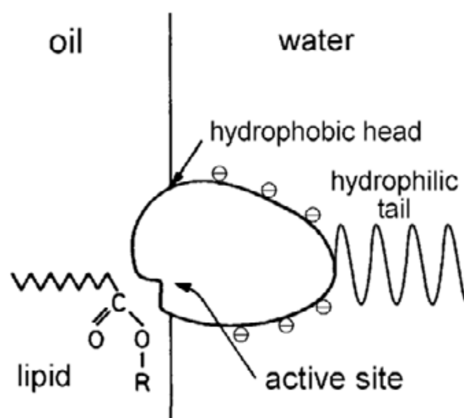

Figure 1.8: Model of pancreatic lipase located at an oil/water interface according to Brockerhoff (1974). Substrate binding site ("active site") and supersubstrate binding site ("hydrophobic head") are adjacent but separate.

Despite a lack of amino acid sequence similarity, all lipases have similar structures (Jaeger & Reetz, 1998). The three-dimensional structure of lipases reveals the characteristic α/β-hydrolase fold. The lipase core is composed of eight parallel β-sheets surrounded and connected by up to six α-helices. The active site of lipases was found to be covered by a surface loop of a hydrophobic oligo-peptide, which is called the 'lid'. The 'lid' moves away on contact with the interface, turning the 'closed' form of the enzyme into an 'open' form. At the same time, this movement increases the hydrophobicity of the surface surrounding the catalytic site, which is thought to facilitate binding of lipase at the interface. Immediately the substrate enters the binding pocket (Brady et al., 1990; van Tilbeurgh et al., 1993). Catalytically active amino acid residues are located in the same positions forming a so-called catalytic triad consisting of a serine, a histidine and an aspartate or glutamate residue, comparable to serine peptidases (e.g. trypsin). The hydrolysis of the substrate is started with a nucleophilic attack of the substrate's carbonyl carbon by the serine oxygen, leading to formation of a tetraedric intermediate (Figure 1.9). The intermediate is stabilized by hydrogen bonds between the negatively charged carbonyl oxygen atom and two main-chain NH groups that belong to the so-called 'oxyanion hole'. The histidine donates a proton to the leaving alcohol component of the substrate. The acid component of the substrate is esterified to the enzyme's serine residue, called covalent acyl-enzyme complex. The deacylation step consists of a nucleophilic attack of the substrate-bound enzyme by water. Again, a tetrahedral intermediate is formed, which is stabilized by interactions with the oxyanion hole. The histidine residue donates a proton to the oxygen atom of the active serine residue thereby releasing the fatty acid product and regenerating the enzyme (Jaeger et al., 1999; Andualema & Gessesse, 2012). Lipase-catalyzed reactions are accelerated by Ca^{2+} ions because the liberated fatty acids are precipitated as insoluble Ca-salts.

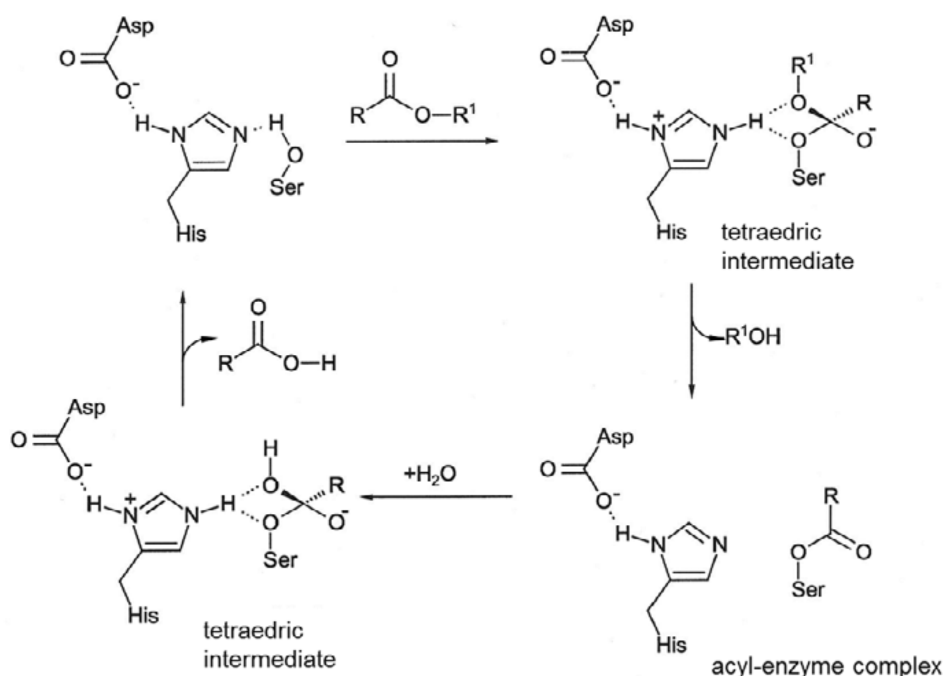

Figure 1.9: Mechanism of lipid hydrolysis by the catalytic triad in lipases.

Three lipase groups can be distinguished based on their substrates. TG lipases (EC 3.1.1.3) act on the ester bonds of TG resulting in release of DG and FFA (Figure 1.10A). They can further hydrolyze ester bonds of MG or glycerol releasing further FFA. Polar lipid hydrolases are denoted as phospholipases or glycolipid hydrolases depending on their substrates. Phospholipases are classified based on the ester bond they cleave within a phospholipid (Figure 1.10B). Phospholipase A_1 (EC 3.1.1.32) has been detected by measuring hydrolysis of PC to LPC (ester bond sn-1) in many cells and tissues from various organism (Aoki et al., 2002). Phospholipases A_2 (EC 3.1.1.4) are the most studied phospholipases and hydrolyze the ester bond on the position sn-2. Phospholipase C (EC 3.1.4.3) cleaves the glycerophosphate bond, while phospholipase D (EC 3.1.4.4) remove the polar head group by leaving the phosphate group attached to glycerol. The third group is the galactolipases (EC 3.1.1.26) which hydrolyze galactolipids and release one or both FFA (Figure 1.10C).

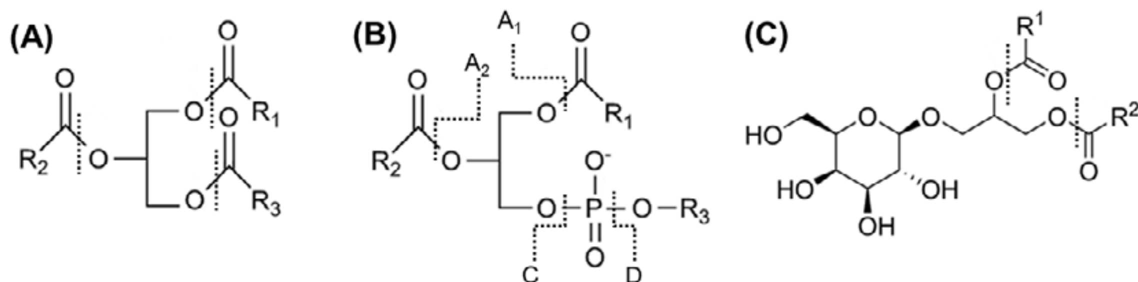

Figure 1.10: Mode of action of different lipase groups. A: Sites of potential hydrolysis by triglyceride (TG) lipases on TG; B: phospholipases on phospholipids, with indication of the various sites attacked for hydrolytic cleavage by the phospholipase types A_1, A_2, C and D; C: galactolipases on galactolipids.

1.3.2 Lipase activity assays

Lipase activity can be measured with fluorochromic substrates, e.g. 4-methyl umbelliferyl fatty acid esters. The generated 4-methyl umbelliferone is quantitated with excitation and emission wavelengths of 365 nm and 445 nm (Jacks & Kircher, 1967). Huggins & Lapides (1947) determined the lipase activity by hydrolysis of p-nitrophenylesters of fatty acids and the release of p-nitrophenol was colorimetrically quantitated at 410 nm. The lipolytic activity can further be determined by employing screening techniques on solid media. Agar plates, containing trioleate or tributyrin as substrate, are widely used to quantitate lipase activity. Lipase products are indicated by formation of clear halos around the colonies (Atlas, 2010). Bacterial colonies show orange fluorescence halos visible on irradiation with an UV lamp at 350 nm on triolein plates containing rhodamine B (Kouker & Jaeger, 1987; Cardenas et al., 2001). The assay is based on the ability of rhodamine B to form colored complexes with the released FFA.

A more laborious but reliable method for identifying the lipase activity is the determination of fatty acids liberated from lipids by titration with NaOH (Jensen, 1983). Lipase activity is proportional to the amount of released FFA. These FFA can also be quantitated using a commercial enzymatic kit (Jaeger & Kovacic, 2014). The enzymatic method relies upon the acylation of coenzyme A by the fatty acids in the presence of added acyl-CoA synthetase. The acyl-CoA is oxidized by added acyl-CoA oxidase with generation of hydrogen peroxide. The presence of peroxidase permits the oxidative condensation of a purple dye which can be quantitated colorimetrically at 550 nm.

Beside lipase activity assays assessing the substrate consumption or the product formation, also the physical properties of the assay system, such as the conductivity, turbidity or interfacial tension can be used. Lipolytic reactions occur at the lipid-water interface and the substrates usually form equilibria between monomeric, micellar and emulsified states (Jaeger & Reetz, 1998). Among the interfacial methods, the monomolecular film technology at the air-water interface has been developed and an oil-droplet technique was adapted for measuring interfacial tension for the purpose of monitoring the lipase activity (Ransac et al., 1999). The lipase kinetics can be monitored by automatic analysis of interfacial parameters, such as surface pressure, molecular area of the substrate or surface coverage of the water-soluble lipases. The decrease of the interfacial tension between the oil and water caused by lipase hydrolysis, is monitored as a function of time (Labourdenne et al., 1997).

The substrate specificity of the lipases, which can vary widely, is essential for the lipase activity. Therefore, different substrates can be hydrolyzed and different fatty acids can be released to a different extent even at the same lipase activity measured toward a standard substrate. However, such lipase activity assays toward a standard substrate are commonly used for standardization of industrial lipase-products, although this activity does not necessarily reveal the real activity in the final application. For example, baking tests for lipases are necessary to determine the optimal dosage. Gerits et al. (2014b) demonstrated that Lipolase and Lecitase Ultra had different impact on bread volume despite of the same activities toward p-nitrophenyl palmitate. Therefore, different lipases have different lipid specificities in wheat dough systems. The level of generated FFA in dough can be a good indicator for lipase activity in the system, but it provides no information of the optimal dosage and the final loaf volume (Gerits et al., 2014b).

1.3.3 Baking lipases

Commercial baked products require uniform consistent performance from all of the ingredients to obtain quality production at the lowest costs. To achieve good quality, the use of emulsifiers, shortenings, and enzymes is common (Casado et al., 2012). Enzyme application in the baking industry is considered as clean label. Exogenous enzymes are being used to improve dough-handling properties, bread volume, crumb softness, crust crispiness, browning, and freshness. The baking industry makes use of different types of amylases,

oxidases, hemicellulases, peptidases, and recently lipases and phospholipases. Synthetic additives can be replaced with these clean label additives. Compared to emulsifiers and chemical agents, which still have a functional effect in the final loaf, it is assumed that enzymes become denatured during baking and have no remaining activity in the final products. This allows enzymes to be clean label improvers and they provide bread improving functions without appearing on the label (Martinez-Anaya & Jimenez, 1997). Thus, partial or complete replacement of traditionally used volume-improving agents is possible. Further, the storage and transportation difficulties of emulsifiers, such as DATEM, and the comparatively lower cost of enzymes is another reason for substitution of emulsifiers. The weight potency of DATEM emulsifier is usually 1/10 or less of that of lipases, which increases the cost of transport (Moayedallaie et al., 2010).

Lipases have been used over the past two decades, along with other enzymes and emulsifiers, to improve some characteristics of baked goods, e.g. to increase bread oven spring and specific volume (Moayedallaie et al., 2010), to improve crumb structure (Poulsen et al., 1998), or to alter bread crust fracture behavior (Primo-Martín et al., 2008). Compared to peptidases and amylases, the use of lipases in the baking industry is quite recent because of their broad substrate specificity and the resulting uncertainty because their lipolytic activities can be beneficial or detrimental.

In the 1990s, the first generation of lipases was introduced to the market. Lipopan 50-BG (Novozymes A/S, Denmark) belonged to the first generation of lipases. It was a purified 1,3-specific lipase from *Thermomyces lanuginosus* produced by submerged fermentation of a genetically modified *Aspergillus oryzae*. These lipases hydrolyze the ester bond between glycerol and fatty acids in positions 1 and 3 of triglycerides, producing FFA, DG, MG and glycerol thereby increasing the amount of lipids with emulsifying properties (Moayedallaie et al., 2010). Lipopan 50 BG strengthened the gluten network, provided improved crumb structure and enhanced dough stability. However, overdosing led to a decrease of loaf volume (Martinez-Anaya & Jimenez, 1997).

The second generation lipases comprised Lipopan F-BG and was developed by Novozymes A/S (Denmark). It is a purified lipolytic enzyme obtained from *Fusarium oxysporum* produced through the submerged fermentation of the genetically modified microorganism *Aspergillus oryzae*. It works on both polar and non-polar lipids in wheat flour, producing

more polar products, such as LPC and DGMG than the first generation lipases. Lipopan F-BG is an example for lipases showing both lipase and phospholipase activity.

Among the third generation lipases Lipopan Xtra-BG is more concentrated and has a better tolerance toward variations in flour type and dosing than Lipopan F-BG. Stojceska and Ainsworth (2008) evaluated the effect of Lipopan Xtra-BG on volume, staling and crumb structure of high fibre white bread. They reported that this enzyme increases expansion of the gluten network, increases the wall thickness and decreases cell density, improving these characteristics of high fibre white bread.

However, the fact is that not all lipases are equally effective in improving bread volume. Ramrakhiani & Chand (2011) suggested that the use of phospholipases might be a better approach to replace emulsifiers than lipases. The perfect baking lipase/phospholipase should have optimal activity on the different lipid substrates available in flour like TG, phospholipids and galactolipids to give the optimal gas bubble stability to replace synthetic emulsifiers (De Maria et al., 2007). Most of the studies on phospholipases in breadmaking have been done by the use of phospholipase type A_2. The commercial phospholipase BakeZyme PH 800 BG (DSM) is a lipolytic enzyme preparation produced by *Aspergillus* species to improve dough and bread characteristics. Panamore Golden (DSM) is a lipolytic enzyme with a dual action on phospholipids and galactolipids to create similar or better effects compared to DATEM. Another commercial microbial phospholipase A_2 tested on the baking market is LysoMax (Du Pont). LysoMax acts on lecithin sublayers with conversion into 2-lysolecithins and FFA. It has been shown that this enzyme can effectively replace DATEM because of similar rheological behavior of the dough, loaf volume increase and improved bread crumb (Sirbu & Paslaru, 2006).

1.3.4 Functionality of lipases in the production of wheat bread

The involvement of lipids and lipid-protein complexes in the stability of dough foam has been studied intensively (MacRitchie, 1976, 1981; MacRitchie & Gras, 1973; Gan et al., 1995). Polar lipids are considered positive. Lipases are useful tools to modify the polarity of lipids. Many baking lipases hydrolyze several substrates, depending on the level and conditions applied. Their effect is generally described in terms of increasing the concentration of polar lipids (Moayedallaie et al., 2010; Colakoglu & Özkaya, 2012), but the interactions of lipids

and gluten proteins, as well as effects of lipases on air inclusion during mixing need to be taken into account (Primo Martin et al., 2006). During dough development, the lipids become trapped or interact with the gluten network by hydrophobic and electrostatic interactions (McCann et al., 2009). Polar lipids from the starch granule surface are transferred to the gluten protein network (Finnie et al., 2010; Gerits et al., 2013). The interaction of lipids and gluten network promotes gluten aggregation by decreasing the electrostatic repulsion between the gluten polymers. This indirectly contributes to gas cell stabilization by increased gluten network strength (Koehler, 2001b; Pareyt et al., 2011; Pomeranz & Chung, 1978) (Figure 1.11).

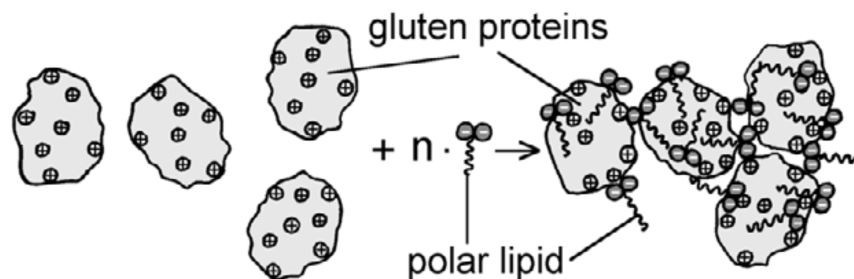

Figure 1.11: Polar and hydrophobic interactions between polar lipids and gluten proteins. Formation of gluten aggregates due to increased number of non-covalent bonds between gluten proteins.

The products of lipase hydrolysis have been claimed to contribute significant emulsifier characteristics and to have improved surface-activity compared to unmodified lipids. The understanding of lipase functionality is continuously being studied, but is mostly based on the knowledge of the effects of lipids in breadmaking. Although no clear description of the mechanism of action of lipases is reported, the generated reaction products have been proposed to provide improvement in dough strength (Poulsen et al., 1998; Primo Martin et al., 2006). In contrast, Colakoglu & Özkaya (2012) suggested that the amount of generated surface-active lipase reaction products is not sufficient to explain greater dough strength, more extensible dough and reduced stickiness. Lipid analysis of the free and bound lipid fractions of fermented dough supplemented with lipases showed that the FFA formed were transferred to the free lipid fraction and the lyso-lipids remained in the bound lipid fraction. This indicates that the FFA do not interact with gluten proteins or that there is only a weak interaction of their hydrophobic fatty acid tail. Consequently, the generated FFA do not contribute to complex formation and gluten strength, while the formed lysolipids interact with

the gluten proteins by their hydrophilic head part. Gerits et al. (2015) suggested that dough rheology is only impacted by the lipid composition readily available at the start of mixing and not by the lipase-reaction products after fermentation. Since the dough rheology examined by Kieffer rig was not affected by lipases, the impact of lipases has to be explained by the direct stabilization mechanism of the liquid film surrounding the gas cells. The liquid film is part of the dough liquor, which should act as medium for incorporation and growth of gas cells in dough (MacRitchie, 1976; Primo Martin et al., 2006; Salt et al., 2006). Increased polar lipid content in dough liquor after incubation with lipases indicated a higher accumulation of these surface-active components at the gas cell interface and, hence, an improved gas cell stabilization (Gerits et al., 2015a).

Because of the increased amount of free polyunsaturated fatty acids after action of lipases, the co-oxidation by wheat lipoxygenase is increased in wheat dough (Castello et al., 1998). This reaction and the surface-active compounds could also contribute to an increased dough strength, possibly by decreasing the surface hydrophobicity of gluten proteins and the dough extensibility by relaxing the strong hydrophobic interactions in the gluten network due to preferential lipid binding to gliadins via hydrophilic bonds (Colakoglu & Özkaya, 2012). The increased dough strength is also correlated with the reduced stickiness of the dough (Collar et al., 1998; Colakoglu & Özkaya, 2012).

Moayedallaie et al. (2010) compared the functional and technological effects different lipase generations with DATEM. All lipases provided bread volume increase after shorter and longer fermentation times, except for Lipopan Xtra. The authors explained the bread volume decrease after long fermentation with Lipopan Xtra by the long action of this highly active lipase on the endogenous lipids, producing stiffer dough. However, Gerits et al. (2014b) showed that an overdose of lipase also caused bread volume decrease. Here, lipid analysis indicated extensive lipid hydrolysis caused by the high activity. Lipopan F and Lecitase Ultra also have a lyso-phospholipase and lyso-galactolipase activity if such substrates are available. Thus, long fermentation times could also lead to further hydrolysis of the generated lyso-phospholipids and lyso-galactolipids leading to volume decrease. This denotes the importance of the optimal lipid composition generated by the breakdown of endogenous lipids to obtain bread volume increase. Moreover, lipases probably catalyze the transition from lipid classes promoting the hexagonal II mesophase to lipid classes promoting the lamellar and hexagonal I mesophases which improve stabilization of gas cells. Overdosing of lipases decreases the level of lipids forming the lamellar mesophase and this does not lead to the further

stabilization of gas cells (Gerits et al., 2014b). Further, overdosing induces earlier oven rise termination due to gas cell coalescence (Gerits et al., 2015a). An optimal dosage of lipase did not extend the oven rise in time, but increased the oven rise height. Altering the endogenous wheat lipids has no impact on starch gelatinization, its enthalpy nor the amylopectin crystal melting (Colakoglu & Özkaya, 2012; Gerits et al., 2015a; Pauly et al., 2012). However, altering the polarity of the endogenous lipids may have impact on gluten protein polymerization during baking (Gerits et al., 2014a). Studies are needed to reveal the impact of lipids and lipases on gluten polymerization during baking.

Until now, it is not clear which lipase currently can be considered as perfect and to what extent each of the lipid classes should be hydrolyzed to provide an optimal functional effect. Further challenges are to unravel the interactions between lipids, lipases and proteins during breadmaking.

Lipase addition has been shown to have an anti-staling effect and to increase bread shelf life (Olesen et al., 2000). The formation of amylose-lipid complexes is accelerated by lipases. MG are more effectively forming amylose-lipid complexes than DATEM and SSL (Ghiasi et al.), indicating that lipases probably play roles in delaying starch retrogradation, mostly in decreasing the initial firmness. Purhagen et al. (2011) showed that lipase addition has no impact on amylopectin retrogradation and, hence, no impact on the firming rate. However, Gerits et al. (2015b) demonstrated that amylopectin retrogradation was slower upon addition of lipases, but in the end proceeded to a similar extent as in the control bread. Thus, lipase hydrolysis products originating from lipids present in the free lipid fraction had probably more impact on amylopectin retrogradation than the FFA and lyso-lipids obtained after hydrolysis from lipids bound to the gluten network.

1.4 Methods to isolate lipid classes and their analytical characterization

1.4.1 Isolation of non-polar and polar lipids from wheat flour

Lipids are extracted from wheat flour or wheat flour products by various methods. General lipid extraction and fractionation in food systems were reviewed (Aoki et al., 2002; Chung et al., 2009; Christie, 2003; Sikorski & Kolakowska, 2010).

Two official solvent extraction methods are used for the extraction of flour lipids. Approved Method 30-25 of AACC International (AACCI 2000) is a semicontinuous Soxhlet extraction using petroleum ether, which facilitates the contact between substrate and solvent. AOCS Official Method Aa-38 (AOCS 2000) is a modification of the procedure using a Butt-tube, which has the advantage of uniform extraction temperature during the process. Both procedures extract nearly the same amount of lipids. The extraction with non-polar solvents like petroleum ether isolates mainly free lipids. For the extraction of bound lipids, polar solvents such as mixtures of alcohol and water, including WSB, are used for stirring and high speed blending methods. Beside chloroform and methanol (2:1), WSB is the commonly used solvent for extraction of free and bound lipids. A drawback of very polar solvents is that carbohydrates and proteins are also extracted to a small extent, which have to be removed by Folch method afterwards (Folch, 1957). This procedure removes the non-lipid material effectively from the lipid extract mixture via a chloroform/methanol/water-phase separation. For extraction of starch lipids hot aqueous alcohols optimized for controlled swelling of starch granules and solubilization of lipids are used. 1-Propanol, 2-propanol or 1-butanol with water can be used under nitrogen at 100 °C. For the quantitation of starch lipids, the use of purified starch samples, isolated from wheat flour, is recommended (Chung et al., 2009).

AOCS has approved the method Am 3-96 (AOCS 2000) for extraction of oil in oilseeds by supercritical fluid extraction. The method uses supercritical CO_2 alone or in combination with 15% ethanol modifier. Hubbard et al. (2004) optimized the method for wheat flour to extract the amount of flour lipids similar to those by the AACC and AOCS methods. Using supercritical fluid extraction, the total lipid yield was higher and more glycolipids, but less non-polar lipids were extracted compared to Soxhlet and Butt methods. The overall benefit is reduction of time, of cost and of exposure to toxic chemicals.

Accelerated solvent extraction belongs to the general category of subcritical fluid extraction (King, 2004). With the accelerated solvent extraction technique, various extraction mixtures can be heated above their boiling point by applying sufficient pressure to keep them liquid. This technique can be considered as an alternative to the classical Folch extraction (Schaefer, 1998). The extraction process is very efficient due to the velocity with which the solvent flows through the sample (Schaefer, 1998).

1.4.2 Fractionation and analytical characterization of wheat flour lipids

For separation of wheat flour lipids into a polar and non-polar fraction, column chromatography, a batch procedure, or both in combination are used. Traditionally, wheat lipids have been separated using silica gel column chromatography with an elution gradient from non-polar to polar. However, column chromatography requires much solvent and time to separate each lipid class based solely on gravity. For smaller amounts of samples, the solid-phase extraction with prepacked columns has some advantages over open-column chromatography, including less solvent consumption and higher recovery. The solid-phase extraction can be used for prefractionation of lipid-extracts. Kaluzny et al. (1985) used aminopropyl-bonded phase columns to separate lipid mixtures into up to ten lipid classes in high yield and purity. Bergqvist and Hersloef (1995) presented a method of extracting DGDG from oat flakes using solid-phase extraction and HPLC to gain pure DGDG. Commercial cartridges were utilized to prepare 100 mg fractions of galactolipids containing more than 97% of DGDG. The small lipid amount and long separation time is a critical point in the characterization of the functional effects of wheat lipids, because reconstitution baking tests of individual lipid classes need clearly higher amounts than can be gained by these methods in acceptable time. De Stefanis and Ponte (1969) described a batch procedure to separate high amounts of wheat flour lipids into a non-polar and polar fraction. Here, the separation of lipid classes with an appropriate solvent was carried out by extraction from the silica gel in a single step procedure in contrast to a continuous elution from the silica gel using an open-column chromatography. This method has the great advantage of being able to prefractionate complex lipid mixtures faster and in bigger quantities than other methods. Selmair (2010) optimized this method to fractionate non polar glyco- and phospholipids of defatted lecithin and oat oil in a preparative scale. Here, up to 30 g lecithin and 57 g oat oil were separated in one batch. Albeit it only being a prefractionation, column chromatography is still needed to obtain pure lipid classes. Selmair (2010) developed a column chromatography to gain pure lipid classes of the lecithin glycolipid mixture obtained from the preceding batch procedure. At the beginning, the non-polar lipids were eluted from the column with chloroform. The remaining polar lipids, glycolipids and phospholipids, had to be further separated by column chromatography. With different mixtures of chloroform/acetone, the glycolipid classes were eluted, while finally the remaining phospholipids were eluted from the column with chloroform/methanol mixtures.

To analyze simply and rapidly lipid mixtures, thin-layer chromatography (TLC) is widely used because of the high separation efficiency and sensitivity. High-performance thin layer chromatography (HPTLC) even improves the analysis through less test volume and a shorter separation time, better resolution and sensitivity. Clayton et al. (1970) used a system with chloroform/methanol/ammonia solution (25%)/water (65/30/5/2.5) and visualized the spots by charring with sulphuric acid (50%). Using this system, the separation and detection of all main lipid classes was possible.

Classical methods for structural elucidation include hydrolysis of the lipids and qualitative and quantitative analysis of the products. Gas chromatography techniques have been mainly used for FFA analysis of wheat lipids, which are usually saponified, converted to fatty acid methyl esters and analyzed by gas chromatography with a flame ionization detector (GC-FID). Phospholipids can be identified by quantitative determination of phosphorous. The proportion of the carbohydrates in glycolipids can be quantitated after acidic hydrolysis by capillary zone electrophoresis with indirect UV detection (Vorndran et al., 1992).

The analytical qualitative and quantitative characterization of lipid mixtures is most commonly performed by high performance liquid chromatography (HPLC) in combination with an evaporation light scattering detector (ELSD). On a conventional normal phase (NP) column, a separation of up to 16 lipid classes in one single run was achieved (Homan & Anderson, 1998). Further improvement was achieved with a polyvinyl alcohol-bonded phase that was used for the separation of sphingolipids, glycoglycerolipids, phospholipids and the more polar lipid constituents (Deschamps et al., 2001). Other authors presented methods for the separation of neutral and polar lipid classes using diol-NP-columns (Silversand & Haux, 1997). They demonstrated excellent separations either for neutral lipids or polar lipids. Selmair (2010) used a HPLC-ELSD system with a diol-column for separation and quantitation of isolated glycolipids.

Graeve and Janssen (2009) established for the first time an improved HPLC method to separate a broad range of lipid classes in one single run on a monolithic silica column using HPLC-ELSD. Gerits et al. (2013) further optimized the method for proper separation and detection of wheat lipids. The optimum gradient and eluent composition allowed separating wheat non-polar and polar lipids and quantitation by internal calibration with cholesterol. However, the ELSD also has disadvantages. Moreau (1994) pointed out that an ELSD requires large amounts of gas for nebulization, while the response of the ELSD is not linear to

the mass of the lipids. Also, the detection of lipids is difficult using gradient with high amounts of water because of irregular baseline. Finnie et al. (2009) quantitated 146 polar lipid species present in wheat whole meal, flour and starch by lipid profiling techniques. The authors used electrospray ionization tandem mass spectrometry (Devaiah et al., 2006) with a sensitivity and resolution previously unattainable, allowing the most complete characterization of wheat polar lipids. For confirmation of structural elucidation, nuclear magnetic resonance spectroscopy (NMR) can be used.

1.5 Methods to evaluate the functional effects of lipid-mixtures

To determine the effects of baking additives, e.g. enzymes, on the production of bakery products various methods including sensory, rheological and baking tests are required to assess the baking performance. Therefore, comparative tests are usually carried out with and without an additive.

1.5.1 Effects on the mixing properties

The first step in breadmaking is the mixing of the ingredients to form a viscoelastic dough. The behavior of the dough with or without additives shows the first effects of enzymes through measuring the consistency of the developing dough in relation to the mixing time with a farinograph. Dough is formed in a prescribed method using a standardized intermeshing Z-blade kneading mixer. Measurement of the mixer shaft torque relates to the apparent viscosity and increases rapidly to a peak before gradually decreasing. The development is displayed in a torque-time diagram (farinogram), whereby the torque is measured as so called Brabender Units (BU). A variety of parameters, like the water absorption of the dough, the dough development time, dough stability, dough softening and dough resistance, can be extracted from the farinogram. The dough development time is the mixing time that is necessary to reach the dough optimum, the maximum of the curve. According to the ICC/AACC standard methods the dough optimum is the point at which the standardized dough reaches a consistency of 500 BU at 30 °C. However, Kieffer et al. (1998) adapted and optimized the standard method to 22 °C instead of 30 °C and a dough optimum of 550 BU ± 10. The lower temperature provides more significant results through a more

distinct curve maximum. The dough stability is defined as the duration of the dough optimum (curve maximum) and the dough softening (mixing tolerance) as the difference between the dough consistency at the curve maximum and the dough consistency at the end of 20 min mixing time. The dough resistance is the sum of dough development time and dough stability. A further important parameter is the water adsorption, which is the amount of water to obtain a consistency of 550 BU at the dough optimum. A micro-farinograph for 10 g flour was developed to examine the effects of additives available only in small amounts.

More recent instruments such as the Mixolab from Chopin and the DoughLab/microDoughLab from Perten Instruments implement the farinograph concept with additional control-of-temperature profile, potentially allowing more insightful interpretation and exploitation of torque curves during dough development and baking (Campbell & Martin, 2012).

1.5.2 Developed dough and gluten

The rheological properties of dough developed to its optimum are examined through tensile tests using an extensograph. Dough strands are extended under standardized conditions until they disrupt. From the resulting force-distance diagram, parameters such as the extensibility, the resistance to extension, and the extension energy can be used to characterize individual wheat varieties or the effects of additives. The same information can be gained for gluten, after washing out the starch with a sodium chloride solution. The force needed to disrupt the strand equals the resistance to extension, the distance until the strand ruptures equals the extensibility and the area under the curve equals the expansion energy of the dough or gluten. The total extension energy strongly correlates with the bread volume (Nash et al., 2006). The higher the value for the extension energy, the higher the bread volume increase will be. The standard test uses 300 g flour. Kieffer et al. (1998) developed a comparable micro-extension test with 10 g flour, which was standardized by Scherf et al. (2016). A high correlation between rheological properties and loaf volume can be obtained, when doughs for extension tests and baking tests are produced with a comparable composition and treatment at the same temperature. It is important that the dough is mixed to its optimal consistency.

1.5.3 Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) is a rather new technique for structural analysis of biological and food material on the µm-scale. It gives the possibility to examine the internal structure of rather thick samples in three dimensions because the CLSM detects in-focus regions only, the out-of-focus parts are appearing black (Dürrenberger et al., 2001). CLSM can be applied to assess the microstructure by a dynamic and nearly non-invasive observation. The acquisition of specific focal sections of the sample enables the visualization of interactions between structural elements without the necessity to prepare the samples by fixing and dehydration. This nearly non-invasive observation facilitates the visualization of cross-links between structural elements or the kinetics of visual changes over time (Jekle & Becker, 2012). However, the micrographs only deliver a section of the microstructure. For the extraction of structural features it is therefore necessary to process and analyze several digital images to obtain structural measures (Jekle & Becker, 2011). CLSM has been proven to be a helpful instrument in cereal science. The component of interest in the dough or bread is stained with a fluorescent dye emitting fluorescence after illumination by light of a specific wavelength. Commonly used fluorescent dyes are rhodamine B for protein staining or nile red for lipid staining. Jekle and Becker (2011) applied a method for quantifying the protein microstructure of wheat dough (DOMIQ; Dough Microstructure Quantification) and established correlations with rheological properties of dough. Highly significant linear correlations between image analysis measurements and rheological attributes were revealed.

1.5.4 Baking activity

To determine the individual baking activity of wheat flour there is still no better way than to evaluate it in baking tests. The standard-scale baking tests are conducted with 1000 g of flour, the normal-scale baking tests with 300-1000 g of flour. With the micro-baking tests, 10 g of flour is sufficient to gain significant results in comparison to the standard-scale baking tests. The micro-baking test (MBT) uses the 10g Farinograph as mixing device. Here, the dough is mixed to its optimum consistency (22 °C, 550 BU). With the micro-rapid-mix test (MRMT) this first step is done in a rapid dough mixing device, at high speed, for a standardized mixing time of 1 min (Selmair & Koehler, 2008) to simulate industrial mixing. Micro-baking tests using 10 g of flour have been shown to provide results that are highly correlated with those

obtained on a standard-scale using 1000 g of flour (Kieffer et al., 1998). Only the absolute volume increases are higher using the micro-baking tests compared to the normal-scale baking tests due to the higher surface-per-weight proportion of 10 g breads than of 300 g / 1000 g breads.

1.5.5 Reconstitution baking tests

The contribution of flour components to baking performance and dough rheology of wheat flour can be investigated using two approaches. One is to add flour fractions to native flour and to determine the effects. For example, Selmair (2010) added different glycolipid classes and samples enriched with glycolipids from different lecithin types to native wheat flour and revealed their baking performance by means of micro-baking and micro-extension tests. The disadvantage of such a method is that the composition of flour containing the additive, in particular the lipid content, is different from the control flour.

An important method of studying the contribution of lipid content or different lipid mixtures in breadmaking was fractionation and reconstitution (Pareyt et al., 2011; Papantoniou et al., 2004; Chung et al., 1977; Chung et al., 1980; Hoseney et al., 1969; MacRitchie & Gras, 1973). With this method, the flour which is investigated has the proximate composition than the control flour. The disadvantages of the fractionation–reconstitution method are that the extraction solvents can have an impact on (other) flour constituents and the individual components might not be present in their native state. In regard to extractability of lipids and recombination of defatted flour plus isolated lipids, proper selection of solvent and extraction temperature is of major importance for extractability of polar lipids and to sustain the functionality of defatted flour. Non-polar solvents, such as diethyl ether, petroleum ether, n-hexane, extract substantially less lipids than more polar solvents (benzene, chloroform, acetone, and especially WSB). Lipids extracted with non-polar solvents contained less polar lipids than those extracted with polar solvents (Finney et al., 1976). However, extracting the lipids with methanol, ethanol or in particular WSB alter the functional properties of flours due to changes in the properties of the gluten fraction of flour (MacRitchie & Gras, 1973) and reduce the gas-retaining capacity of gluten proteins (Finney et al., 1976). Further, WSB forms complexes with starch (Schoch, 1942) and alters its functionality. Hoseney et al. (1969) have attempted to overcome this problem by extracting free lipids from flour with petroleum ether

and then by extracting the bound lipids from isolated lyophilized gluten. The reconstituted flour had restored rheological properties after premixing. However, the more common way for defatting and subsequent reconstitution tests is to use chloroform or dichloromethane as extraction solvent since they do not alter the rheological properties of the flour components. Chung et al. (1977) suggested to extract flour lipids with 2-propanol at 75 °C to maximize lipid extraction and minimize damage to breadmaking properties of reconstituted flours. All in all, the problem of the complete extraction of free and bound non-starch lipids and subsequent reconstitution experiments with suitable defatted flour has not yet been solved.

1.5.6 Crumb softness and anti-staling effect

Loss of oven freshness during storage of bread encompasses a number of different changes in the product. Assessing firmness can give an indication of the amount of physical staling that has occurred in the product and is measured using a compression test. A typical compression test will compress the sample through a given distance and measure the force required to achieve a given percentage thickness compression. From the force-distance diagram the maximal force needed to deform the crumb is correlated with the crumb firmness. Additionally the shape of the curve can be used to obtain information on the pores and at which point they collapse (Selmair, 2010).

Physical staling is the intrinsic firming of the bread crumb and is mainly caused by the retrogradation of the starch. The crumb softness decreases rapidly and the bread becomes wrinkly and hard. The magnitude and rate of the retrogradation process depend on a number of formulation factors and the conditions of storage. In addition, a firmness test can be used to assess the effect that different ingredients and additives might have on the staling process (Young, 2012).

1.6 Research requirements in the field of lipases in breadmaking

It was anticipated that lipases would soon out-compete artificial emulsifiers due to several advantages as already described. However, the use of emulsifiers is still unexpectedly wide-spread and the market for emulsifiers is still growing. The reason for this is that emulsifiers still are considered to induce superior stability to doughs and to be less influenced by different

applications and raw material compared to lipases. For example, low pH (< 4.9) and mechanical stress acting on the dough during industrial bread production is very challenging for lipase-containing doughs as opposed to doughs containing e.g. DATEM, which is known for its stabilizing effect on the dough matrix under various conditions.

Currently available baking lipases hydrolyze a number of different lipid structures in the flour and individual lipases mainly prefer specific lipid classes as substrates. This means that the action of lipases in dough results in formation of a range of products and a detailed characterization of these lipase reaction products would be an important step in understanding the mechanism of lipases in baking applications and thus an important step in developing improved baking lipases. However, quantitative data of the lipid composition affected by different baking lipases are not available. Until now, it is not clear which lipase can currently be considered as perfect and to what extent each of the lipid classes should be hydrolyzed for optimal performance. The fact is that not all lipases are equally effective in improving the bread volume. Moreover, overproofing and overdosing of baking lipases are also critical factors which can negatively influence the quality of the baked good. This denotes the importance of the optimal lipid composition after breakdown of endogenous lipids to obtain bread volume increase.

The understanding of lipase functionality is continuously being studied, but is mostly based on the knowledge of the effects of lipids in breadmaking. Although no clear description of the mechanism of action of lipases is reported, the impact of lipases has to do with the direct stabilization mechanism of the liquid film surrounding the gas cells. However, further research is needed to confirm this hypothesis and to understand the technological effects of lipase-reaction products on a molecular level. The decisive mode of action to explain their structure-function relationship of specific lipid classes has still not been conclusively revealed. Up to date there is little knowledge on how the lipase reaction products unfold their techno-functional properties during breadmaking, nor is it known how the molecular structure of the lipid classes is correlated with the bread volume.

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2. Aim of the work

Due to the research requirements in the field of baking lipases outlined before, the aim of this study was to quantitate lipids from wheat flour and wheat dough after addition of different commercial lipases. Systematic fractionation-reconstitution studies should be made to unravel the functional effects of lipase-modified wheat lipids in breadmaking. Further, the relationships between specific wheat lipid classes and their functional effects for the baked good were to be determined. Altogether, the optimal hydrolytic profile of lipases in relation to baking was supposed to be elucidated and insights into the mechanism of action of baking lipases should be gained from a molecular point of view.

To solve these aims it was necessary to establish a HPLC-ELSD method to separate all polar and non-polar wheat lipid classes, as well as the lipase reaction products. Quantitation of wheat lipids required obtaining reference compounds either by isolation from wheat lipids or by lipase-aided hydrolysis from isolated compounds and fractions. The functional effects of lipases in baking should be demonstrated by micro-baking tests using 10 g of flour and classical surfactants such as DATEM and SSL were used as benchmarks.

The next core prerequisite of this work was to develop a method for fractionation and reconstitution of wheat flour to investigate the functional effects of lipase-treated wheat lipids by means of micro-scale methods. The study should be focused on the production and characterization of fully functional defatted flour suitable for recombination with lipase-treated dough lipids. The influence of the defatting method on the technological properties and the gluten network of flour should be determined by rheological and analytical tests.

To determine the relation between lipid mixtures and their functional effects in wheat breadmaking, combinations of polar and non-polar lipids as well as of specific isolated wheat lipid classes should be quantitated by HPLC-ELSD and analyzed for their functional effects using the established micro-reconstitution baking test.

3. Results

3.1 Lipases in wheat breadmaking: analysis and functional effects of lipid reaction products

The baking activity of two different lipases was evaluated by a micro-baking test on a 10 g flour basis and the altered lipid composition of lipase-treated wheat lipids was quantitated. Monika Schaffarczyk partly designed the experiments, she performed the experiments, collected and evaluated the data.

For identification and quantitation of the various lipid classes, several pure glycolipids and phospholipids were isolated from wheat lipids by a silica gel batch procedure and silica gel column chromatography. Using these references, Monika Schaffarczyk established an improved high-performance liquid-chromatography method with evaporative light scattering detection using a monolithic column, which was able to separate and quantitate all wheat lipid classes and lipase reaction products. For quantitation of the lipid classes, cholesterol was used as internal standard.

Monika Schaffarczyk isolated wheat lipids, dough lipids and dough lipids after lipase addition by extraction with WSB (20 °C) and 2-propanol (75 °C). It was clearly shown that the former was the preferred solvent for lipid isolation. Furthermore, the data showed that specific lipid classes were hydrolyzed and the concentration of the corresponding reaction products increased. Especially DGDG (-0.9 mmol/kg flour), MGDG (-0.4 mmol/kg), and NAPE (-0.3 mmol/kg) were hydrolyzed and a concomitant formation of digalactosyl monoglycerides (DGMG) (+0.6 mmol/kg), monogalactosyl monoglycerides (MGMG) (+0.6 mmol/kg), and N-acyl-lysophosphatidyl ethanolamine (NALPE) (+0.5 mmol/kg) was found.

Some of the products, in particular galacto- and phospholipids, are supposed to improve the baking performance, whereas others such as free fatty acids have negative effects on the baking properties. Therefore, the type and concentration of the added lipase appears to be of major importance. Micro-baking tests showed bread volume increases of 56-58% depending on the type and concentration of the added lipase. The current results confirm the important relationship between lipid composition and baking performance of flour.

Additionally, Monika Schaffarczyk wrote the manuscript and revised it according to the comments of the reviewers.

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Lipases in Wheat Breadmaking: Analysis and Functional Effects of Lipid Reaction Products

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S *Supporting Information*

ABSTRACT: The baking activity of two different lipases was evaluated by a microbaking test on a 10 g flour basis, and the altered lipid composition of lipase-treated wheat lipids was quantitated. To identify and quantitate the various lipid classes, pure glycolipids and phospholipids were isolated from a wheat flour lipid extract by a silica gel batch procedure and silica gel column chromatography. These reference compounds were used to establish a high-performance liquid chromatographic method with evaporative light scattering detection, which was able to separate all of the wheat lipid classes and lipase reaction products. Wheat lipids, dough lipids, and dough lipids after lipase addition were quantitated using cholesterol as an internal standard. Especially digalactosyl diglycerides (−0.9 mmol/kg flour), monogalactosyl diglycerides (−0.4 mmol/kg), and *N*-acyl-phosphatidyl ethanolamine (−0.3 mmol/kg) were hydrolyzed, and a concomitant formation of digalactosyl monoglycerides (+0.6 mmol/kg), monogalactosyl monoglycerides (+0.6 mmol/kg), and *N*-acyl-lysophosphatidyl ethanolamine (+0.5 mmol/kg) was found. The lipase-induced changes of the lipid fraction caused increases in bread volume of 56−58%, depending on the type and concentration of the added lipase. The current results confirm the important relationship between the lipid fraction composition and the baking performance of flour.

KEYWORDS: *lipase, wheat lipids, HPLC-ELSD, microbaking test*

■ INTRODUCTION

The lipid content of wheat is considered of utmost importance for the baking performance of wheat flour. Wheat grain usually contains 3−4% lipid depending on genetics and growth conditions, while wheat flour contains 1−2.5% lipid depending on the raw materials and milling conditions. Wheat flour lipids are divided into two major groups based on their location and functionality. Two-thirds are nonstarch-bound lipids and one-third is starch-bound lipids.[1] Starch lipids are primarily composed of lysophospholipids, which form inclusion complexes with amylose helices in native starch.[2] Only the nonstarch lipids are considered to be active in baking.[3] Nonstarch lipids are extractable at room temperature (RT) and can be further subdivided into free and bound lipids according to the solvent used for their extraction.

Although lipids are present in wheat in considerably lower levels than are starch and protein, they exhibit important functional properties in breadmaking. Several studies have examined the functionality of endogenous wheat flour lipids in breadmaking based on fractionation and reconstitution methods, as reviewed in detail by Pareyt et al.[4] It was also shown that the improved bread properties were mainly due to the polar wheat lipids,[5,6] and glycolipids are considered more active than phospholipids as suggested by De Stefanis and Ponte.[7]

In the past few decades, demands for consistent quality in baked goods led to the application of a wide range of additives in the baking industry. Beside synthetic emulsifiers such as diacetyl tartaric esters of mono- and diglycerides (DATEM) or sodium stearoyl-2-lactylate (SSL), lipases along with other enzymes are being used to improve the baking quality of wheat flour. Currently available lipases for baking applications hydrolyze a number of different lipid structures in flour and lead to improved surface activity of these endogenous lipids, thus resulting in the significant increase in bread volume after baking.[10] The specific improving effect of individual lipases is mainly considered to be due to their preference for certain lipid classes, resulting in the formation of a lipase-specific range of products.[8] In particular, this has been shown by Gerits et al.,[9,10] who also suggested theories on the mechanism of action. Altogether, information about lipase-generated lipids is known to some extent for single components, but in-depth understanding of lipase-mediated reaction products is missing, and especially information regarding the function of lipid mixtures generated by the action of lipases is still very scarce.

The aim of this study was to quantitate lipids from wheat flour and wheat dough after the addition of two different commercial lipases. Therefore, the generation of lipase reaction products and the consumption of the lipase substrates were quantitatively analyzed. Different lipase concentrations as well as two different lipid extraction procedures were used. The functional effects of lipases in baking were demonstrated by microscale baking tests using 10 g of flour, and classical surfactants such as DATEM and SSL were used as benchmarks. Quantitation of wheat lipids required obtaining reference compounds either by isolation from wheat lipids or by lipase-aided hydrolysis from isolated compounds and fractions. High

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performance liquid chromatography coupled to an evaporative light scattering detector (HPLC-ELSD) based on a method published by Gerits et al.[9,10] was applied for lipid analysis with the aim to separate all polar and nonpolar wheat lipid classes, as well as the lipase reaction products, in a single HPLC run. In contrast to Gerits et al.,[10] absolute concentrations of lipid classes were determined.

■ MATERIALS AND METHODS

Wheat Flour. 'Kolibri' flour, a commercial flour obtained from a mixture of wheat cultivars (Meneba, Rotterdam, The Netherlands, 2012 harvest), was characterized as follows. The moisture and ash contents of the flour were determined according to ICC Standards 110[12] and 104,[13] respectively. Nitrogen contents were determined by means of the method of Dumas on a TruSpec N nitrogen analyzer (Leco, Kirchheim, Germany). A conversion factor of 5.7 was used to calculate the crude protein content from the nitrogen content. Analytical characteristics of the flour were 13.3% moisture, 10.7% protein (dry mass), and 0.52% ash (dry mass).

Chemicals and Reagents. Fresh baker's yeast was obtained from Wieninger GmbH (Passau, Germany). All solvents used were HPLC or LC−MS grade and from Sigma-Aldrich (Steinheim, Germany). The following reagents were purchased from the sources given in squared brackets: L-α-phosphatidic acid sodium salt from chicken egg (PA), L-α-phosphatidyl ethanolamine from chicken egg (PE), L-α-phosphatidyl choline (95%) from chicken egg (PC), L-α-phosphatidyl serine sodium salt from soy (PS), L-α-phosphatidyl inositol sodium salt from soy (PI), monogalactosyl diglyceride (MGDG), digalactosyl diglyceride (DGDG), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate sodium salt (LPA), L-α-lysophosphatidyl choline from soy (LPC), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phospho ethanolamine (LPE), L-α-phosphatidyl glycerol sodium salt from soy (PG), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phospho-(1′-rac-glycerol) sodium salt (LPG), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phospho-L-serine sodium salt (LPS), L-α-lysophosphatidyl inositol sodium salt from soy (LPI) [Avanti Polar Lipid through Otto Nordwald GmbH, Hamburg, Germany]; steryl glucosides (SG), acylated steryl glucosides (ASG), ceramides (*N*-acyl-sphingosine) (CER), glucocerebrosides (cerebroside) (CBR) [Matreya LLC through Bio Trend, Cologne, Germany]; Monomuls 90−35 (monoglycerides containing 75% stearic and 35% palmitic acid, monoglyceride content 90%), Lametop 300 (80% DATEM, 20% calcium carbonate as anticaking reagent); Prefera SSL 6000 [BASF, Illertissen, Germany]; glyceryl-1,3-distearate, glyceryl trioleate, ethanolamine (>99%), acetic acid eluent additive for LC−MS [Sigma-Aldrich, Steinheim, Germany]; silica gel G 60 (0.04−0.063 mm), silica gel (0.063−0.200 mm), concentrating zone-high performance thin layer chromatography (HPTLC) plates (20 × 10 cm^2) coated with silica gel G 60 on glass, concentrating zone-HPTLC plates (20 × 20 cm^2) coated with silica gel G 60 on glass [VWR Merck, Darmstadt, Germany]; commercial enzyme granulate Lipopan F-BG (25.0 KLU/g), commercial enzyme granulate Lipopan Xtra-BG (7.2 KLU/g) [Novozymes A/S, Bagsvaerd, Denmark]. *N*-acyl phosphatidyl ethanolamine and monogalactosyl monoglyceride were obtained from Novozymes (Bagsvaerd, Denmark).

Lipid Extraction from Flour. Two different lipid extraction methods were compared regarding the extracted lipid composition. Lipids were extracted from flour using water-saturated 1-butanol (WSB) or 2-propanol at 75 °C. WSB was obtained when HCl (1 L; 80 mmol/L) and 1-butanol (2.5 L) were stirred for 15 min. Subsequently, the water phase was removed using a separating funnel. For each extraction, flour (1000 g) and WSB (3.5 L) were stirred at RT for 16 h. After centrifugation (20 min, 4 °C, 3550 g), the solvent was removed and collected by filtration on a glass filter (porosity 3, pore size 16−40 μm). The combined extracts were evaporated to dryness on a rotary evaporator (Büchi, Rotavapor R-210). The defatted flour was spread out on a flat glass tray in a fume hood for one week. The second extraction method used 2-propanol at 75 °C as the extraction solvent according to Chung et al.[14] The defatted flour was dried as described for the WSB-defatted flour.

Lipid Extraction from Dough. The dough was prepared from 50 g of flour by upscaling the recipe for 10 g of flour used by Köhler and Grosch.[15] The ingredients were as follows: flour (50 g), water (27.2 mL), NaCl (1.0 g), sucrose (0.5 g), fresh baker's yeast (3.5 g), L-*threo* ascorbic acid (20 mg/kg flour), and lipase (0−170 mg/kg flour; Lipopan F-BG or Lipopan Xtra-BG, Novozymes, Bagsvaerd, Denmark). The flour, NaCl, sucrose, baker's yeast, and lipase were premixed dry for 1 min in a Farinograph (50 g Z-blade mixer, Brabender, Duisburg, Germany) at 22 °C. Ascorbic acid solution (1.5 mL, 0.67 g/L) and water (27.2 mL) were added within 25 s and mixing was continued until the optimum consistency of the dough (550 Brabender units at 7 min) was reached. The dough was divided into two equal pieces, one was immediately frozen with liquid N_2, and the other one was allowed to rest (20 min, 30 °C, water-saturated atmosphere). The dough was then reshaped on a dough rounder (Type 440, Brabender, Duisburg, Germany) for 10 cycles, and the resulting spherical dough piece was rolled (PTFE cylinder; diameter, 5 cm; length, 30 cm) to yield an oval dough piece of 5 mm thickness. The dough piece was folded twice to $^1/_4$ of its original size and was reshaped on the dough rounder for 20 cycles. After proofing (30 °C, water-saturated atmosphere, 38 min), the dough was also frozen in liquid N_2. Both dough pieces were freeze-dried and milled using an ultracentrifugal mill ZM 200 (200 μm mesh size, Retsch, Haan, Germany).

Lipids were extracted from the freeze-dried dough powder (10.0 g) by the powder being stirred with WSB (40 mL, 16 h, RT) or 2-propanol (80 mL, 2 h, 75 °C). After centrifugation (5 min, 4 °C, 3550 g), the supernatant was filtered (0.45 μm), and the solvent was evaporated to dryness using a rotational vacuum concentrator (Christ, Osterode, Germany) and stored under an Ar atmosphere at −75 °C until further analysis.

Isolation of Lipid Reference Compounds. Reference compounds for the identification and quantitation of lipid classes by HPTLC and HPLC-ELSD were isolated from wheat flour by WSB-extraction. The lipids (6 g) were prefractionated using a preparative solid-phase extraction protocol for complex lipid mixtures ("batch procedure") according to Selmair and Koehler,[6] and all batches were examined by thin layer chromatography.[6] A crude glycolipid fraction was obtained, which was further separated by column chromatography to obtain three pure glycolipid classes, ASG, MGDG, and DGDG, as reported by Selmair and Koehler[6] and three further fractions containing mixtures of lipid classes. The latter three fractions (mixtures of SG/MGDG, NAPE/DGDG, and NAPE/NALPE/PC/DGMG) were further purified by column chromatography. The separation of the fraction containing SG and MGDG was performed by column chromatography using activated silica gel. The silica gel (G 60, 0.040−0.063 mm) was activated as follows: 200 g of silica gel were dried overnight at 105 °C and left to cool in a desiccator. After the addition of water (10 mL), the material was shaken for 20 min. The activated silica gel was dispersed in dichloromethane/acetone/2-propanol (60/39/1, v/v/v), poured into a glass column, and packed to a height of 38 cm (diameter 2 cm). The sample was dissolved in 2 mL of chloroform and applied to the column. The lipids were eluted with 1 L of dichloromethane/acetone/2-propanol (60/39/1, v/v/v). Each fraction was evaporated and examined by HPTLC (see above). Fractions comprising the same pure lipid class were recombined, evaporated to dryness, and weighed.

The fraction containing NAPE and DGDG was separated by silica gel chromatography (35 × 2 cm^2) in the same manner as described above using the eluent dichloromethane/methanol (87/13, v/v; 600 mL) and dichloromethane/methanol (83/17, v/v; 400 mL).

To obtain pure NALPE, the fraction containing NAPE, NALPE, PC, and DGMG was separated using column chromatography in the same manner as described above. The eluents were dichloromethane/methanol (87/13, v/v; 1 L), dichloromethane/methanol (80/20, v/v; 500 mL), and methanol (200 mL).

Synthesis of Digalactosyl Monoglyceride (DGMG). Isolated DGDG (105 mg) was emulsified in phosphate buffer (5 mL, pH 6.9, 50 mmol/L) with a T8 Ultra-Turrax (IKA Labortechnik, Staufen) and Lipopan F (5.0 mg) was added. The suspension was incubated for 2 h

at 30 °C in a thermo shaker (HLC BioTech, Bovenden, Germany). The lipids were extracted with chloroform (4 × 5 mL) and separated by activated silica gel column chromatography (3 × 27 cm^2) using dichloromethane/methanol (500 mL 84/16, v/v; 500 mL 82/18, v/v; 500 mL 77/23, v/v; 200 mL methanol) as the mobile phase. Each fraction was evaporated and examined by HPTLC (see above). Fractions containing DGMG were combined and evaporated to dryness.

Nuclear Magnetic Resonance (NMR) Spectroscopy. The ^1H and two-dimensional NMR spectra (^1H, COSY ($^1H^1H$, correlated spectroscopy), HMQC ($^1H^{13}C$, heteronuclear multiple quantum coherence), and HMBC ($^1H^{13}C$, heteronuclear multiple bond correlation)) of NALPE and DGMG were recorded on a Bruker AMX 400 II Ultrashield spectrometer (400 MHz, Bruker Biospin, Rheinstetten, Germany) and the ^{13}C NMR spectra were recorded on a Bruker AMX 360 spectrometer (360 MHz, Bruker Biospin, Rheinstetten, Germany). All of the experiments were conducted at a temperature of 25 °C. Chemical shift values δ (in ppm) are given relative to the signal for internal tetramethylsilane (TMS, $\delta = 0$). The values for coupling constants J are given in Hz (see Supporting Information).

HPLC-ELSD Analysis of Lipid Mixtures. A HPLC-ELSD analysis was conducted on a 100 × 2 mm Chromolith Performance Si column with a 5 × 4.6 mm security guard Chromolith precolumn (Merck, Darmstadt, Germany). A Jasco HPLC (PU-1580; Gross-Umstadt, Germany) was used in combination with a Jasco autosampler (AS-2057 Plus), a Jasco HPLC Pump (PU-1580), a Jasco Ternary Gradient Unit (LG-1580−02), and a ThermaSphere TS-130 column oven (Phenomenex, Aschaffenburg, Germany) set at 40 °C. The detector was a Sedex 85 ELSD (Sedere, Alfortville, France). The carrier gas used was compressed air with a pressure of 0.35 MPa. The evaporation chamber was kept at 40 °C, the gain was set at 3 or 8, and for the computerized analysis, the software KromaSystem 2000 (Version 1.60) was used.

The gradient of the HPLC solvent was based on the method of Graeve and Janssen[11] for marine lipids. Gerits et al.[9] adapted the procedure for wheat flour lipids to improve the separation of the polar lipid classes; however, further optimization was carried out to separate all wheat flour lipids and lipase reaction products. Table 1 lists the mobile phase solvent gradient. The composition of the three solvent mixtures and the gradient were adapted to improve the separation of glycolipids and phospholipids since not all lipid classes were separated with the gradient of Gertis et al.[9] With the optimized procedure, all nonpolar lipids, glycolipids, and phospholipids were separated in one single run, but for quantitation, two runs at different gain settings were necessary to get signals suitable for calculation. Calibration curves were generated from the isolated or purchased lipid reference compounds, respectively. Two standard mixtures with concentrations of 0.05 μg/μL, 0.1 μg/μL, 0.2 μg/μL, and 0.3 μg/μL of each reference compound were prepared. The solvent was chloroform/methanol (50/50, v/v) containing cholesterol as the internal standard (250 mg/L) as suggested by Gerits et al.[10] The first standard mixture consisted of FFA, MG, CER, SG, NAPE, NALPE, and DGMG; the second mixture contained DG, CBR, ASG, MGDG, MGMG, PC, and LPC. The calibration curves for the lipid classes were obtained by injecting the standard mixtures (10 μL) and monitoring the ELSD-signal at gain 8. The calibration curves of TG and DGDG, which were present in high concentrations, were acquired at a low sensitivity (gain 3) with absolute amounts of TG and DGDG of 5, 10, 15, and 20 μg. The calibration curves were plotted as the peak area ratio (sample/cholesterol) versus the amount of each sample. In all of the calibration and quantitative separations, the inject volume was 10 μL, thus cholesterol was always present in the same amount while the amounts of the analytes varied.

The extracted lipids from flour or dough were dissolved (5 mg lipid extract/mL) in chloroform/methanol (50/50, v/v) containing cholesterol as an internal standard (0.25 mg/mL). The samples (10 μL) were injected into the HPLC column (10 μL) and analyzed by HPLC-ELSD. The ELSD-signal was monitored at gain 3 for TG and DGDG and at gain 8 for all other lipid classes (one run per gain setting).

Table 1. Ternary Gradient of Mobile Phase Used for the HPLC Separation of Lipid Classes from Wheat

time (min)	flow rate (mL/min)	% solvent A[a]	% solvent B[a]	% solvent C[a]
0.0	1.4	100	0	0
1.6	1.4	98	2	0
4.5	1.4	96	4	0
8.0	1.4	80	10	0
14.0	1.4	65	15	20
14.1	1.4	55	25	20
19.0	1.4	70	0	30
27.0	1.4	65	0	35
27.1	3.0	0	100	0
28.5[b]	3.0	0	100	0
30.0	3.0	100	0	0
32.0	3.0	100	0	0
34.0	1.4	100	0	0
35.0	1.4	100	0	0

[a]A, isooctane; B, acetone/ethyl acetate/2-propanol (54/38/8, v/v/v) containing 0.02% (v/v) acetic acid; C, 2-propanol/water (85/15, v/v) containing 0.02% (v/v) acetic acid and 0.05% (v/v) ethanolamine. [b]End of analysis, start of the column wash.

Microbaking Test (MBT). The microbaking test using 10 g of flour was conducted as described by Köhler and Grosch.[15] The appropriate amount of water was determined by a modification of the ICC standard procedure 115 (22 °C instead of 30 °C).[16] Flour (10 g), NaCl (0.2 g), sucrose (0.1 g), baker's yeast (0.7 g), and different amounts of lipases (30−170 mg/kg) or surfactants (0.2−0.8%) were premixed dry for 1 min in a microfarinograph (10 g Z-blade mixer, Brabender, Duisburg, Germany) at 22 °C. An L-*threo*-ascorbic acid solution (0.3 mL, 0.67 g/L) and water (5.44 mL) were added within 25 s and the mixing was continued until the optimum consistency of the dough (550 BU at 7 min) was reached. After removal from the mixer, the dough was fermented (20 min, 30 °C, water-saturated atmosphere). The dough was then reshaped on a dough rounder (custom-made) for 10 cycles. The resulting dough ball passed through the rolls (3 mm roll-gap) of an AMPIA-pasta machine (model 150 mm—Deluxe, Marcato, Italy) to form an oval dough piece. This piece was folded twice to $^1/_4$ of its original size and reshaped on the dough rounder for 20 cycles. The dough was then proofed (30 °C, water-saturated atmosphere, 38 min) and baked (180 °C increasing to 250 °C, 10 min) on an automatic proofing−baking line.[5] The volume of the bread was determined using a benchtop laser-based device (VolScan Profiler, Stable Micro Systems, Godalming, U.K.).

Micro-Rapid-Mix Test (MRMT). The dough was microbaked with 10 g of flour and mixed at high-speed for 1 min according to Köhler and Grosch[15] except that the proofing and baking was done in-line with an automatic proofing-baking device. The recipe and additives were the same as described for the MBT.

Statistical Analysis. Triplicate determinations were carried out for each control bread and each concentration of additive. The data was statistically evaluated using Microsoft Office Excel 2010 (Microsoft Corporation, Seattle, WA). The following values were used to rate the levels of significance: $p > 0.05$, not significant; $p < 0.05$, statistically significant; $p < 0.01$, significant; and $p < 0.001$, highly significant. The statistical evaluation of all quantitative data was carried out via one-way analysis of variance (ANOVA) using the software Sigma Plot 11.0 (Systat, San José, CA).

RESULTS AND DISCUSSION

Reference Compounds for the Quantitation of Wheat Lipid Classes. Quantitative methods require reference

Journal of Agricultural and Food Chemistry Article

compounds for calibration and all relevant lipid classes from wheat are to be available. For this purpose, pure lipid classes were isolated from wheat flour lipids to obtain reference compounds for HPLC-ELSD analysis. WSB-extracted wheat lipids were prefractionated by a batch procedure into a nonpolar lipid fraction and two polar fractions.[6] The latter were a crude glycolipid and a phospholipid fraction. The glycolipid fraction was further separated by column chromatography into pure fractions of ASG (yield: 4.7%, 103 mg), MGDG (yield: 8.3%, 182 mg), and DGDG (yield: 29%, 650 mg). However, further fractions contained mixtures of lipid classes; therefore, SG, CBR, MGMG, NAPE, NALPE, and DGMG could not be isolated with this column. Thus, mixtures of these compounds had to be further purified by column chromatography.

Nonstarch wheat lipids contain an average of 3% DGMG, but the isolated amount was not sufficient for subsequent purification. Hence, the isolated DGDG was hydrolyzed to DGMG by incubation with Lipopan F at 30 °C in a phosphate buffer. After purification by silica gel column chromatography, DGMG was obtained in a yield of 30% (32 mg). The byproduct MGMG was also isolated with a yield of 8% (9 mg) because impurities of MGDG present in the initial DGDG-fraction were also hydrolyzed by the lipase. These pure glycolipid classes were identified by HPTLC[5] and HPLC-ELSD having used authentic reference compounds commercially available in small amounts. The identity of DGMG and NALPE were verified by NMR because no reference compounds were commercially available. NMR data and structures of DGMG and NALPE are given in the Supporting Information.

HPLC-ELSD Method to Quantitate Wheat Lipid Classes. For wheat lipid analysis, an optimized HPLC-ELSD procedure with a ternary gradient of the mobile phase was used. The gradient was optimized in numerous steps to be able to separate all relevant lipid classes that were considered (Table 1). A typical chromatogram of wheat lipids is shown in Figure 1. Only the separation of LPC was complicated because of the irregular baseline at this part of the chromatogram. This affected the accuracy of the quantitative determination of this compound. Table 2 shows the retention times of the 27 reference compounds that were separated in one single run. The different classes of wheat lipids were present in a wide range of concentrations (e.g., ~40% TG vs ~3% PC based on total wheat lipids) so that two different sensitivities of the ELSD (separate runs for gain 3 and gain 8) were necessary to get peaks suitable for quantitation of all of the wheat lipid classes. The relative responses (i.e., signal intensity per mass unit of lipid) of the different lipid classes varied according to the lipid type and the injected amount so that nonlinear relationships between the ratio of the signal intensities (sample/internal standard) and the ratio of the quantities (sample/internal standard) were obtained. Depending on the lipid class, different polynomial calibration curves were fitted from as many calibrators as possible (0.5−3.0 μg (gain 8), 5−20 μg (gain 3)).

Lipid Composition of Flour and Dough Lipids without Lipase Addition. Table 3 shows the quantitated lipid classes of wheat lipids extracted from 'Kolibri' flour and fermented dough as affected by the solvent used for lipid isolation. On one hand, wheat flour lipids were extracted using 2-propanol at 75 °C (yield: 1.57 ± 0.03% dm) to minimize flour damage and to maximize lipid extraction. On the other hand, WSB was used at RT with a yield of 1.67 ± 0.05% dm. The quantitated lipid composition of wheat flour is in accordance with the literature,[1,17] with TG as the major component of nonpolar

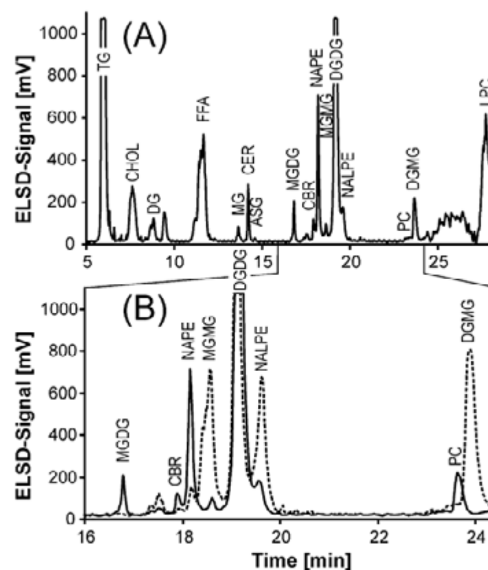

Figure 1. HPLC chromatograms of wheat flour lipids (solid line) and dough lipids after mixing and fermentation in the presence of lipase (Lipopan Xtra 130 mg/kg) (dashed line). (A) Retention time of 5−29 min and (B) retention time of 16−24.5 min with details of the relevant lipid classes. Lipid classes are designated as defined in Table 2. Lipids were extracted with WSB at RT.

Table 2. Retention Times of Lipid Classes from Wheat after Separation by HPLC

abbreviation	lipid class	retention time (min)
TG	triglycerides	5.1
CHOL	cholesterol	7.6
DG	diglycerides	8.6
FFA	free fatty acids	11.4
MG	monoglycerides	13.5
CER	ceramides	13.7
ASG	acylated sterol glucosides	14.5
SG	sterol glucosides	16.6
MGDG	monoglactosyl diglycerides	16.8
CBR	cerebrosides	17.5
NAPE	*N*-acyl phosphatidyl ethanolamine	18.1
MGMG	monogalactosyl monoglycerides	18.5
DGDG	digalactosyl diglycerides	19.1
PE	phosphatidyl ethanolamine	19.5
NALPE	*N*-acyl lysophosphatidyl ethanolamine	19.6
PG	phosphadyl glycerol	20.0
PA	phosphatidic acid	21.7
PI	phosphatidyl inositol	22.9
PS	phosphatidyl serine	23.3
PC	phosphatidyl choline	23.4
DGMG	digalactosyl monoglycerides	23.8
LPE	lysophosphatidyl ethanolamine	24.0
LPI	lysophosphatidyl inositol	24.3
LPG	lysophosphatidyl glycerol	24.6
LPS	lysophosphatidyl serine	24.9
LPA	lysophosphatidic acid	25.3
LPC	lysophosphatidyl choline	28.3

Journal of Agricultural and Food Chemistry Article

Table 3. Concentration of Lipids from Wheat Flour and Wheat Dough after Proofing (58 min) as Affected by the Addition of the Lipase Lipopan F and the Solvent Used for Lipid Extraction[a]

lipid class[b]	concentration (mg/kg dry matter)				
	flour lipids	dough lipids with lipase added (mg/kg flour)			
		0	30	70	110
Lipid Extraction Using 2-Propanol (75 °C)					
TG	$5391^{A} \pm 14$	$2948^{B} \pm 219$	$3043^{B} \pm 100$	$3040^{B} \pm 160$	$2649^{BC} \pm 140$
FFA	$1910^{A} \pm 416$	$1046^{B} \pm 187$	$1189^{B} \pm 152$	$1570^{A} \pm 90$	$1591^{A} \pm 118$
DG	$458^{A} \pm 27$	$283^{B} \pm 39$	$203^{B} \pm 23$	$203^{B} \pm 11$	$181^{BC} \pm 2$
MG	$179^{A} \pm 13$	$90^{B} \pm 15$	$83^{B} \pm 7$	$85^{B} \pm 15$	$83^{B} \pm 11$
ASG	$89^{A} \pm 7$	$73^{A} \pm 7$	$74^{A} \pm 5$	$102^{B} \pm 3$	$100^{B} \pm 11$
SG	$26^{A} \pm 10$	$16^{A} \pm 4$	$19^{A} \pm 4$	$33^{A} \pm 9$	$18^{A} \pm 7$
CBR	$278^{A} \pm 4$	$170^{B} \pm 5$	$204^{B} \pm 20$	$240^{B} \pm 20$	$219^{AB} \pm 42$
DGDG	$1929^{A} \pm 40$	$1370^{B} \pm 282$	$1356^{B} \pm 14$	$1209^{C} \pm 110$	$970^{C} \pm 39$
DGMG	$157^{A} \pm 24$	$177^{A} \pm 11$	$132^{A} \pm 22$	$337^{B} \pm 14$	$312^{B} \pm 64$
MGDG	$529^{A} \pm 55$	$373^{A} \pm 96$	$207^{B} \pm 49$	$171^{B} \pm 42$	$87^{B} \pm 71$
MGMG	$180^{A} \pm 4$	$103^{A} \pm 66$	$194^{A} \pm 53$	$520^{B} \pm 41$	$399^{B} \pm 10$
NAPE	$779^{A} \pm 35$	$578^{A} \pm 120$	$393^{B} \pm 58$	$342^{B} \pm 29$	$207^{C} \pm 29$
NALPE	$443^{A} \pm 35$	$426^{A} \pm 18$	$500^{A} \pm 91$	$602^{B} \pm 59$	$651^{B} \pm 66$
PC	$153^{A} \pm 32$	$145^{A} \pm 34$	$219^{A} \pm 61$	$164^{A} \pm 80$	$144^{A} \pm 44$
LPC	$245^{A} \pm 70$	$340^{A} \pm 190$	$822^{A} \pm 313$	$830^{A} \pm 250$	$907^{A} \pm 350$
Lipid Extraction Using Water-Saturated 1-Butanol (20 °C)					
TG	$5383^{A} \pm 150$	$4248^{B} \pm 118$	$3945^{C} \pm 33$	$3108^{D} \pm 187$	$4266^{BD} \pm 231$
FFA	$1979^{A} \pm 90$	$1512^{B} \pm 117$	$1915^{A} \pm 104$	$2250^{A} \pm 195$	$2791^{AC} \pm 100$
DG	$444^{A} \pm 30$	$426^{A} \pm 40$	$263^{B} \pm 5$	$185^{B} \pm 3$	$226^{B} \pm 27$
MG	$165^{A} \pm 20$	$168^{A} \pm 30$	$159^{A} \pm 14$	$107^{A} \pm 6$	$120^{A} \pm 42$
ASG	$79^{A} \pm 4$	$78^{A} \pm 28$	$79^{A} \pm 31$	$62^{A} \pm 2$	$86^{A} \pm 27$
SG	$28^{A} \pm 12$	$18^{A} \pm 6$	$19^{A} \pm 6$	$34^{A} \pm 6$	$33^{A} \pm 6$
CBR	$284^{A} \pm 11$	$283^{A} \pm 12$	$294^{A} \pm 17$	$264^{A} \pm 17$	$224^{A} \pm 32$
DGDG	$2143^{A} \pm 160$	$2043^{A} \pm 320$	$1949^{A} \pm 207$	$1209^{B} \pm 40$	$1148^{B} \pm 91$
DGMG	$234^{A} \pm 20$	$180^{A} \pm 10$	$295^{A} \pm 72$	$487^{B} \pm 120$	$540^{B} \pm 116$
MGDG	$501^{A} \pm 20$	$498^{A} \pm 25$	$271^{B} \pm 40$	$144^{C} \pm 3$	$158^{C} \pm 18$
MGMG	$304^{A} \pm 11$	$328^{A} \pm 14$	$623^{B} \pm 83$	$563^{B} \pm 1$	$663^{B} \pm 18$
NAPE	$1071^{A} \pm 30$	$1013^{A} \pm 3$	$950^{A} \pm 128$	$729^{B} \pm 4$	$742^{B} \pm 53$
NALPE	$561^{A} \pm 30$	$524^{A} \pm 74$	$884^{B} \pm 43$	$851^{B} \pm 6$	$841^{B} \pm 59$
PC	$190^{A} \pm 90$	$244^{A} \pm 148$	$205^{A} \pm 11$	$191^{A} \pm 135$	$210^{A} \pm 27$
LPC	$400^{A} \pm 180$	$520^{A} \pm 250$	$842^{A} \pm 261$	$936^{A} \pm 350$	$950^{A} \pm 223$

[a]Mean values ± standard deviations of triplicate determinations. Mean values associated with different capital letters are significantly different within each lipid class (one-way ANOVA, Tukey test, $p \leq 0.05$) [b]Lipid classes are designated as defined in Table 2.

lipids, DGDG of glycolipids, and NAPE of phospholipids. As mentioned before, natural fluctuations of the lipid composition of wheat depend on the individual genetic predisposition (cultivar), the agricultural practices used during cultivation, the environmental effects to which the plant was exposed, and the age of the milled flour.

The lipid compositions per kilogram of dry flour extracted with different solvents differed mainly in the polar lipid composition. More glycolipids and phospholipids were extracted using WSB than using 2-propanol (75 °C). It is known that beside free lipids, bound lipids are also extracted almost completely with WSB,[4] which is possibly due to the "bipolar" character of this solvent. WSB is a combination of a nonpolar and a very polar compound, which is more similar to polar lipids than it is to (water-free) 2-propanol, and thus, dissolves these compounds more effectively. This might explain the higher polar lipid portion in the WSB compared to the 2-propanol-extracted lipids, because bound lipids are more efficiently detached from their binding sites.[4,9] The amount of each lipid class was lower in the dough lipids than in the flour lipids when 2-propanol was used as the extraction solvent. In contrast, the compositions of flour and dough lipids were not significantly different in the lipid extracts obtained with WSB. Our results are in line with the phenomenon of "lipid binding".[4,14] This means that when flour is wetted and mixed into dough, around 70% of the total flour lipids become bound to or are entrapped by the gluten network.[9] Because of the binding of lipids during mixing, fewer lipids were extracted with 2-propanol from dough than were extracted from flour. These results suggest WSB as the preferred solvent for total lipid extraction from wheat flour and dough.

Baking Performance as Affected by the Addition of Lipase. To reveal the maximum bread volume increase caused by the addition of lipases and their dose/volume increase dependency, two versions of a microscale baking test based on 10 g of flour differing in the time and intensity of mixing were applied.[18] In the micro-rapid-mix test (MRMT), the dough was mixed for 2 min with a high-speed mixer (1250 rpm), whereas in the microbaking test (MBT), the dough was mixed in a Microfarinograph (63 rpm) until the optimum consistency of the dough was reached (7 min). All other steps were identical. To take dough losses during breadmaking into account, the loaf volumes were based on the dough weight (mL/g dough) before the second proofing session. The bread volume was expressed

Journal of Agricultural and Food Chemistry — Article

in relation to the respective mean value of the control breads in the individual test series, taking into account both climatic and technical fluctuations.

The breads were baked using the MRMT to study the lipase-dependent volume increase after the addition of Lipopan Xtra (10−70 mg/kg). The lipase was applied as a solid or as a watery suspension; the latter procedure was used to get a more homogeneous distribution of the enzyme. However, both application forms did not cause a significant increase of the bread volume. Even after the application of 700 and 1400 mg of Lipopan Xtra per kilogram of flour, no volume increase was obtained. Similar results were obtained for Lipopan F (50 and 500 mg/kg). Also, for the Lipopan F addition, no significant increase in the loaf volume was obtained after the proofing time was extended from 37 to 54 min. Recent studies[8,19] show that Lipopan F and Lipopan Xtra caused a significant volume increase when a normal scale baking test (300 g of flour) with dough mixing in the Farinograph was used. Consequently, the MBT was used to demonstrate the effect of added lipases. It is known that a rather high-volume increase is typical of the microscale setup when compared to conventional baking. Previous studies on the effect of DATEM have shown that the volume increases were higher in the microscale baking test (40−60% volume increase) than in a normal-scale test with 300 g of flour (10−20% volume increase).[20] However, the correlation between the micro- and normal-scale baking tests was high because the same trend of the loaf volumes was obtained with both setups.[18,20] As shown in Figure 2, significant

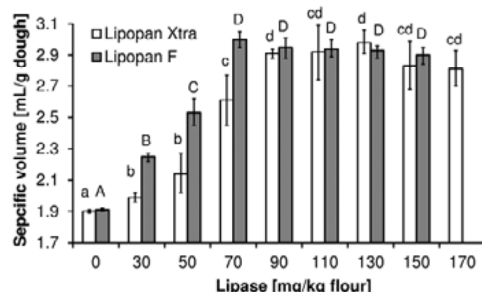

Figure 2. MBTs with 10 g of flour. Change of the bread volume as affected by the increasing concentrations of the lipases Lipopan Xtra and Lipopan F. Error bars represent the mean standard deviations of triplicate determinations. Mean values associated with different letters are significantly different within each lipase concentration (one-way ANOVA, Tukey test, $p \leq 0.05$).

volume increases were obtained for both Lipopan Xtra and Lipopan F. The volume increase was found to correlate to the enzyme dosage until a plateau was reached. Lipopan F was found to reach a plateau at a lower dosage (70 mg granulate/kg flour) than did Lipopan Xtra (90 mg granulate/kg flour). A further increase in the enzyme dosage did not cause statistically significant differences in the obtained bread volumes.

These results strongly suggest the MBT as the preferred test to assess the effects of lipase addition. It can only be speculated as to why the MRMT was not suited to study the functional effect of lipases. One explanation would be that the enzymes were shear sensitive and were inactivated by the high mechanical input during mixing.

The commercial synthetic surfactants DATEM and SSL were chosen as reference surfactants to evaluate the potential of lipases and lipase-treated lipids in breadmaking. To determine the concentration of surfactants for a maximum increase in the bread volume, concentrations of 0.2−0.8% were applied. DATEM was applied as a solid or as a watery suspension. There were no significant differences between the two application forms, and a maximum volume increase of 58% at a concentration of 0.6% was obtained. The typical plateau phase was seen at DATEM concentrations between 0.4 and 0.8%, comparable with the plateau phase after lipase application. SSL showed the same phenomenon and caused a maximum bread volume increase of 47% at a concentration of 0.4%. In summary, the functional effects of lipases (volume increase of 56−58%) were comparable to those of DATEM (58%) and superior to those of SSL (47%). Furthermore, the amount of commercial lipase product added, concentration of lipase required for this effect, was approximately 1/50 of the amount of surfactants added.

Changes of Lipid Composition as Affected by Lipase Addition. Figure 1 shows an overlay of the chromatograms of the wheat flour lipids and lipase-treated dough lipids. It can be clearly seen that especially certain polar lipid classes (retention time 15−25 min) were hydrolyzed after lipase addition. The quantitative data of the lipase-treated dough lipids are given in Tables 3 and 4. In the following sections, the lipid classes TG, DGDG, NAPE, and DGMG are used as examples to discuss the effect of lipases in more detail. Lipase-treated (Lipopan F and Lipopan Xtra) dough lipids differed significantly from the untreated dough lipids and the flour lipids. The results also confirmed that WSB was more effective in extracting the relevant lipid classes than was 2-propanol. With an increase in the amount of lipases, a decrease of TG, DGDG, NAPE, and MGDG occurred, in particular after proofing. This went hand in hand with the increased concentrations of the hydrolysis products FFA, MGMG, NALPE, and DGMG.

Upon review of the composition of WSB extracted lipids, TG were hydrolyzed by lower concentrations of Lipopan F than of Lipopon Xtra, and the concomitant formation of FFA was observed. This corresponds to Lipopan Xtra having a higher affinity toward phospholipids than does Lipopan F. Furthermore, a larger decrease of DGDG (2043 → 1148 mg/kg flour) and the corresponding increase of DGMG (180 → 540 mg/kg) were detected after proofing with Lipopan F than with Lipopan Xtra (DGDG, 2043 → 1350 mg/kg; DGMG, 180 → 265 mg/kg). However, the changes of the DGDG and DGMG concentrations (Tables 3 and 4) revealed that the formation of DGMG did not compensate for the decrease of the DGDG concentration. An explanation could be that DGMG is a transient intermediate and is subjected to further hydrolysis by the lipase. In contrast to DGDG, the addition of Lipopan Xtra resulted in a significantly stronger hydrolysis of MGDG (498 → 26 mg/kg) and NAPE (1013 → 661 mg/kg) after proofing than did the addition of Lipopan F (MGDG, 498 → 158 mg/kg; NAPE, 1013 → 742 mg/kg). Lipid concentrations before fermentation are given in the Supporting Information (Tables S3 and S4) and show the same trends as the concentrations after proofing.

To get a closer overview of the degradation and formation of lipase substrates and products, mass concentrations were converted into molar concentrations. This was done exemplarily for the addition of Lipopan F and for WSB as a lipid extraction solvent (Table 3). Molar masses of the lipid classes were calculated by the use of linoleic acid (M = 280.5 g/mol) as the default fatty acid. The results for the relevant lipid classes

Journal of Agricultural and Food Chemistry — Article

Table 4. Concentration of Lipids from Wheat Flour and Wheat Dough after Proofing (58 min) As Affected by the Addition of the Lipase Lipopan Xtra and the Solvent Used for Lipid Extraction[a]

	concentration (mg/kg dry matter)				
		dough lipids with lipase added (mg/kg flour)			
lipid class[b]	flour lipids	0	90	130	170
		Lipid Extraction Using 2-Propanol (75 °C)			
TG	5391^{A} ± 14	2948^{B} ± 219	2548^{B} ± 300	3044^{B} ± 269	2893^{B} ± 122
FFA	1910^{A} ± 416	1046^{B} ± 187	1184^{B} ± 31	1613^{AC} ± 51	1626^{AC} ± 18
DG	458^{A} ± 27	283^{B} ± 39	226^{B} ± 46	209^{B} ± 19	196^{B} ± 16
MG	179^{A} ± 13	90^{B} ± 15	93^{B} ± 24	79^{B} ± 3	92^{B} ± 1
ASG	89^{A} ± 7	73^{A} ± 7	75^{A} ± 33	54^{A} ± 16	68^{A} ± 12
SG	26^{A} ± 10	16^{A} ± 4	12^{A} ± 1	18^{A} ± 3	16^{A} ± 3
CBR	278^{A} ± 4	170^{B} ± 5	195^{B} ± 17	204^{B} ± 6	213^{B} ± 31
DGDG	1929^{A} ± 40	1370^{B} ± 282	1372^{B} ± 80	1129^{C} ± 71	1051^{C} ± 42
DGMG	157^{A} ± 24	177^{A} ± 11	170^{A} ± 3	195^{A} ± 79	307^{B} ± 64
MGDG	529^{A} ± 55	373^{A} ± 96	55^{B} ± 15	14^{B} ± 1	19^{B} ± 15
MGMG	180^{A} ± 4	103^{A} ± 66	276^{B} ± 14	372^{C} ± 8	412^{D} ± 12
NAPE	779^{A} ± 35	578^{A} ± 120	227^{B} ± 77	156^{B} ± 4	224^{B} ± 43
NALPE	443^{A} ± 35	426^{A} ± 18	576^{B} ± 82	569^{B} ± 7	685^{C} ± 65
PC	153^{A} ± 32	145^{A} ± 34	206^{B} ± 10	190^{B} ± 32	187^{B} ± 30
LPC	245^{A} ± 70	340^{A} ± 190	361^{A} ± 135	644^{A} ± 221	762^{AB} ± 70
		Lipid Extraction Using Water-Saturated 1-Butanol (20 °C)			
TG	5383^{A} ± 150	4248^{B} ± 118	3191^{C} ± 360	3619^{C} ± 214	3750^{C} ± 58
FFA	1979^{A} ± 90	1512^{B} ± 117	1827^{B} ± 70	1826^{B} ± 90	2635^{C} ± 120
DG	444^{A} ± 30	426^{A} ± 40	258^{B} ± 36	245^{B} ± 41	282^{B} ± 28
MG	165^{A} ± 20	168^{A} ± 30	170^{A} ± 55	153^{A} ± 10	164^{A} ± 29
ASG	79^{A} ± 4	78^{A} ± 28	69^{A} ± 5	117^{A} ± 31	106^{A} ± 33
SG	28^{A} ± 12	18^{A} ± 6	29^{A} ± 11	30^{A} ± 2	35^{A} ± 1
CBR	284^{A} ± 11	283^{A} ± 12	249^{A} ± 11	237^{A} ± 15	252^{A} ± 33
DGDG	2143^{A} ± 160	2043^{A} ± 320	1441^{B} ± 70	1526^{B} ± 200	1350^{B} ± 67
DGMG	234^{A} ± 20	180^{A} ± 10	170^{AB} ± 55	140^{AB} ± 20	265^{B} ± 108
MGDG	501^{A} ± 20	498^{A} ± 25	168^{B} ± 16	42^{C} ± 41	26^{C} ± 88
MGMG	304^{A} ± 11	328^{A} ± 14	639^{B} ± 5	620^{B} ± 45	657^{B} ± 98
NAPE	1071^{A} ± 30	1013^{A} ± 3	711^{B} ± 154	609^{B} ± 125	661^{B} ± 54
NALPE	561^{A} ± 30	524^{A} ± 74	807^{B} ± 7	781^{B} ± 60	902^{B} ± 92
PC	190^{A} ± 90	244^{A} ± 148	184^{A} ± 52	239^{A} ± 22	199^{A} ± 30
LPC	400^{A} ± 180	520^{A} ± 250	831^{AB} ± 64	938^{B} ± 58	990^{AB} ± 350

[a]Mean values ± standard deviations of triplicate determinations. Mean values associated with different capital letters are significantly different within each lipid class (one-way ANOVA, Tukey test, $p \leq 0.05$). [b]Lipid classes are designated as defined in Table 2.

are shown in Figure 3. The dough without added lipase contained almost equal molar concentrations of TG and FFA (5.5−6 mmol/kg flour). Upon the addition of Lipopan F, TG decreased by 2.5 mmol/kg flour to a minimum value of 3.5 mmol/kg flour (Figure 3A). The corresponding product FFA strongly increased by 4.5 mmol/kg flour to a final concentration of 10 mmol/kg flour as the lipase concentration increased. Thus, hydrolysis of TG only explained 56% of the FFA formation, clearly showing that other substrates such as glyco- and phospho-lipids also contributed to FFA formation.[10] The hydrolysis of NAPE and the concurrent formation of NALPE (Figure 3B) were approximately in the same range (−0.3 vs +0.5 mmol/kg flour, respectively). Comparable results were obtained from the hydrolysis of MGDG (−0.4 mmol/kg flour) and the formation of MGMG (+0.6 mmol/kg flour) (Figure 3D). The situation was somewhat different for DGDG and DGMG (Figure 3C). The decrease of DGDG (−1 mmol/kg flour) was twice as high as the increase of DGMG (+0.5 mmol/kg flour). A possible reason was given above. The calculation of the balance of the reactions on a molar basis showed that the total increase of FFA after lipase addition (+4.5 mmol/kg flour) was almost equaled by the decreases of the lipase substrates DGDG, MGDG, NAPE, and TG. Hydrolysis of these substrates explained 93% of the lipase-mediated formation of FFA (4.2 of 4.5 mmol/kg flour).

Relevance of the Modified Lipid Composition for the Baking Performance. Nonpolar lipids and NAPE have a negative influence on baking performance, whereas all glycolipids show positive baking potential.[6] Consequently, the hydrolysis of NAPE to NALPE can be regarded as positive for the baking performance. The formation of MGMG from MGDG is also desirable because it was found that the latter has the proper ratio between hydrophilic and lipophilic moieties in the molecular structure to yield good functionality.[6]

Glycoglycerolipids containing two carbohydrate moieties show different behavior compared to one carbohydrate moiety glycoglycerolipids. Previous studies have shown that DGDG and MGMG have comparable positive effects in baking.[6] Thus, the second acyl residue in DGDG must be essential for a high functionality of this lipid class in baking, while a weaker effect has been shown for purified DGMG.[6] Moreover, these studies have shown that DGDG had the best functional effects of all glycolipids. As a consequence, the degradation of DGDG found here (Tables 3 and 4) can be considered unfavorable because

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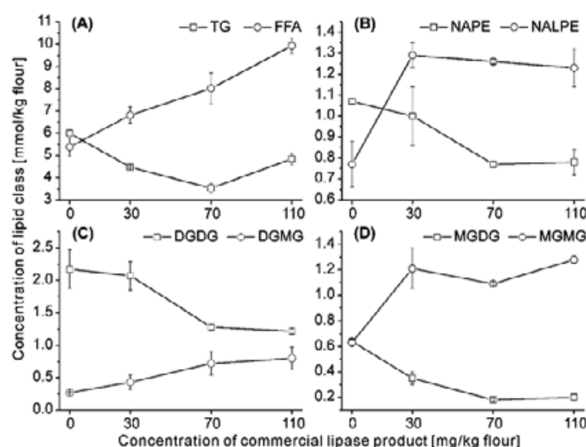

Figure 3. Decrease of selected lipase substrates and increase of the corresponding products as affected by the addition of Lipopan F. Mass concentrations given in Table 3 were converted into molar concentrations [mmol/kg flour] using linoleic acid as the default fatty acid. Lipid classes are designated as defined in Table 2. Lipids were extracted with WSB at RT.

the functional effects of DGDG are much stronger than those of DGMG. Generally, the increased concentration of FFA from lipolytic action is considered to decrease the positive effect of the addition of lipases. Experiments in which free linoleic acid (0.1%, 3.6 mmol/kg) was added, show a considerable decrease in the loaf volume (data not shown).

A comparison of the quantitative data of the lipase-modified lipids (Tables 3 and 4) with bread volumes (Figure 2) as affected by lipases shows that the highest amount of polar hydrolysis products obtained at the highest levels of Lipopan F (110 mg/kg) and Lipopan Xtra (170 mg/kg) did not match the concentrations that caused maximum bread volumes (70 and 130 mg/kg, respectively). Accordingly, a higher concentration of polar lipids did not automatically cause a higher bread volume, although the bread volume remained on a high level compared to the bread volume obtained without the addition of lipase. This effect can be explained by the increased amounts of FFA and DGMG, which have a negative (FFA) or at least a less positive effect (DGMG) on the baking performance. Therefore, the type and concentration of the added lipase appears to be of major importance. Evidence for this hypothesis is given in the work of Gerits et al.,[10] who showed that the specificity of the added lipase clearly affects its technological effect.

Finally, synergistic effects between glycolipids and phospholipids must also be considered. Tables 3 and 4 as well as Figure 2 show that PC was also hydrolyzed to LPC. It is generally accepted that lysophospholipids exhibit stronger functional effects than do phospholipids.[21,22] Furthermore, Wilde et al.[23] indicated that the complex of LPC with the lipid-binding protein puroindoline A is more surface active than is LPC or puroindoline A alone, suggesting a synergistic effect in breadmaking. In addition, already very low concentrations of glycolipids are able to enhance the functionality of phospholipids significantly. Selmair[24] showed that a small amount of MGMG added to the phospholipids of lecithin resulted in an improved baking performance of the flour.

■ ASSOCIATED CONTENT

Supporting Information

NMR data and structures of DGMG and NALPE. Quantitative data of lipid classes present in dough lipids after mixing (without fermentation). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

ASG, acylated sterol glucosides; BU, Brabender Units; CBR, cerebrosides; CER, ceramides; DG, diglycerides; DATEM, diacetyl tartaric acid ester of mono- and diglycerides; DGDG, digalactosyl diglycerides; DGMG, digalactosyl monoglycerides; dm, dry matter; FFA, free fatty acids; HPLC-ELSD, high performance liquid chromatography coupled with an evaporative light scattering detector; HPTLC, high performance thin layer chromatography; KLU, kilo-lipase-units; LPA, lysophosphatidic acid; LPC, lysophosphatidyl choline; LPE, lysophosphatidyl ethanolamine; LPI, lysophosphatidyl inositol; LPS, lysophosphatidyl serine; MBT, microbaking test; MG, monoglycerides; MGDG, monogalactosyl diglycerides; MGMG, monogalactosyl monoglycerides; MRMT, microrapid-mix-test; NALPE, N-acyl-lysophosphatidyl ethanolamine; NAPE, N-acyl-phosphatidyl ethanolamine; NMR, nuclear magnetic resonance spectroscopy; PA, phosphatidic acid; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; PS, phosphatidyl serine; SD, standard deviation; SG, sterol glucosides; TG, triglycerides; WSB, water-saturated 1-butanol

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SUPPLEMANTARY MATERIAL

Table S1. ^{1}H, HMQC and ^{13}C data of N-acyl lysophosphatidyl ethanolamine (NALPE) obtained by lipase hydrolysis of N-acyl phosphatidylethanolamine in chloroform-d

Carbon atom	**Chemical shift (ppm), multiplicity, coupling constant (Hz)**	
	^{1}H	**^{13}C**
Acyl moiety		
1’	-	177.6
2’/ 2”	2.32 t / 2.18 t	34.0 / 35.4
3’	1.58 m	24.6
4’-7’	1.26 m	29.3
8’	2.04 m	27.2
9’, 13’	5.37 m	130.2
11’	2.76 t	25.6
10’, 12’	5.33 m	127.9
14’	2.04 m	27.2
15’	1.26 m	29.7
16’	1.27 m	31.5
17’	1.30 m	22.6
18’	0.89 m	14.1
Glycerol moiety		
1a	3.95 m	64.3
1b	4.10 m	
2	4.01 m	68.5
3a	3.86 m	66.9
3b	3.99 m	
Ethanolamine moiety		
4	3.72 m	59.9
5	3.42 m	39.5

Table S2. ^{1}H, HMQC and ^{13}C data of digalactosyl monoglyceride (DGMG) obtained by lipase hydrolysis of digalactosyl diglyceride (DGDG) in methanol-d_4

Carbon atom	Chemical shift (ppm), multiplicity, coupling constant (Hz)	
	^{1}H	^{13}C
Acyl moiety		
1’	-	173.8
2’	2.38 t	33.5
3’	1.65 m	24.6
4’-7’	1.36 m	28.2
8’	2.08 m	26.7
9’, 13’	5.34 m	129.7
11’	2.79 t	25.0
10’, 12’	5.34 m	128.0
14’	2.08 m	26.7
15’	1.39 m	28.2
16’	1.39 m	31.4
17’	1.39 m	22.3
18’	1.39 m	13.0
Glycerol moiety		
1	4.17	64.6
2	5.06 m	72.8
3a	3.74 m	68.1
3b	4.00 dd	
Carbohydrate moiety A”		
1”	4.27, d, 7.2	103.9
2”	3.54 m	70.8
3”	3.51 m	72.5
4”	3.92 m	66.5
5”	3.72 m	60.8
6” a	3.77 m	68.7
6” b	3.89 m	
Carbohydrate moiety B”		
1”	4.89, d, 3.5	99.0
2”	3.79 m	68.8
3”	3.70 m	68.7
4”	3.94 m	66.6
5”	3.85 m	70.2
6”	3.80	61.1

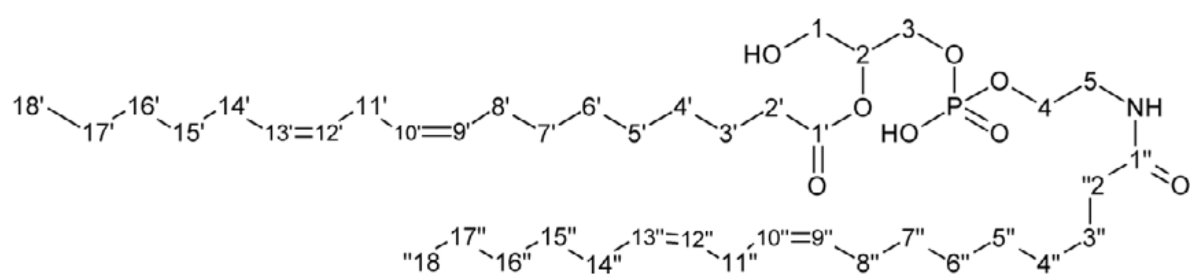

Figure S1. Structure of N-acyl lysophosphatidyl ethanolamine (NALPE)

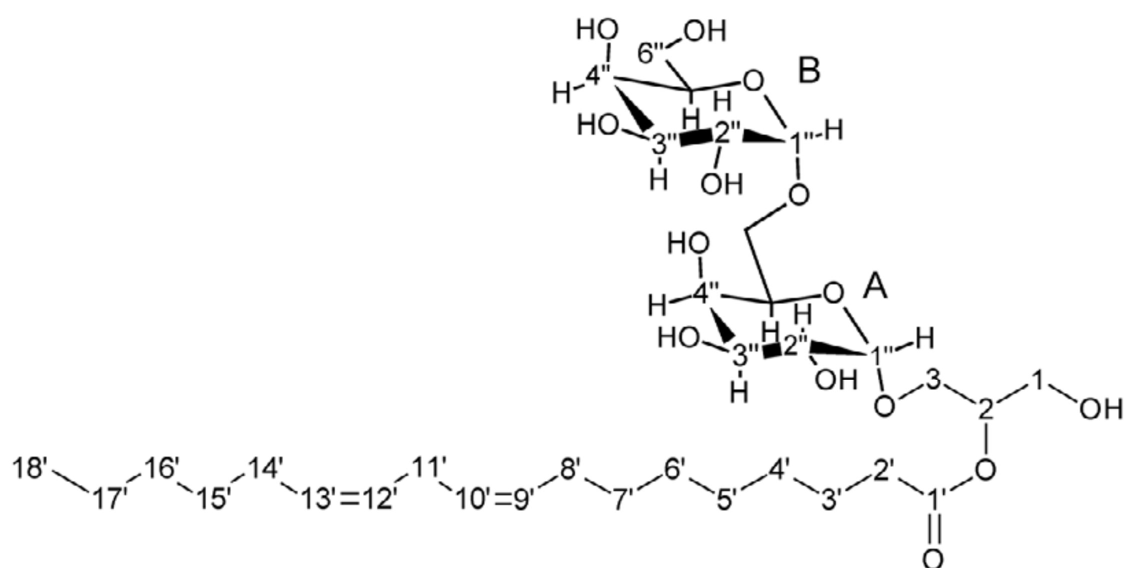

Figure S2. Structure of digalactosyl monoglyceride (DGMG)

Table S3. Concentration of lipids from wheat dough after mixing (7 min) without proofing as affected by the addition of the lipase Lipopan F and the solvent used for lipid extraction[a].

Lipid class[b]	Concentration [mg/kg dry matter]			
	Dough lipids with lipase added [mg/kg flour]			
	0	30	70	110
	Lipid extraction using 2-propanol (75 °C)			
TG	3035±222	2903±134	2654±141	3090±104
FFA	957±110	1549±126	1053±158	1312± 37
DG	258± 54	269± 68	187± 18	230± 50
MG	121± 25	67± 7	214± 15	177± 29
ASG	72± 6	55± 1	111± 5	80± 15
SG	29± 14	11± 2	16± 5	17± 11
CBR	161± 13	190± 26	149± 2	196± 18
DGDG	1463±304	1569±316	1572±119	1457±114
DGMG	168± 62	108± 76	130± 14	135± 4
MGDG	434± 85	222± 29	197± 42	153± 71
MGMG	81± 14	114± 9	154± 34	153± 8
NAPE	493±116	559± 94	444± 60	478± 80
NALPE	384± 93	416± 6	421± 42	537± 54
PC	174± 79	121± 28	213± 80	221± 44
LPC	330± 97	354±113	390±250	451±250
	Lipid extraction using water-saturated n-butanol (20 °C)			
TG	4601±298	4736±109	4198± 32	4413±108
FFA	1531±104	1575± 64	910± 29	1028±461
DG	378± 11	321± 10	232± 31	304± 24
MG	159± 36	186± 4	110± 3	128± 42
ASG	100± 26	103± 23	65± 2	77± 22
SG	40± 5	47± 3	33± 2	29± 8
CBR	245± 67	271± 16	232± 13	254± 12
DGDG	2067±317	1915±176	1597±145	1290±192
DGMG	96± 50	84 ± 72	115± 60	336±100
MGDG	429± 53	415± 23	290± 40	297± 67
MGMG	290± 34	410± 41	479± 42	600± 88
NAPE	1055± 77	969± 29	834± 60	870± 30
NALPE	639±145	726± 30	648± 60	841±156
PC	174± 48	355± 19	270± 75	139± 25
LPC	500±232	808± 51	869±275	976±140

[a] mean values ± standard deviations of triplicate determinations

[b] TG, triglycerides; FFA, free fatty acids; DG, diglycerides; MG, monoglycerides; ASG, acylated sterol glucosides; SG, sterol glucosides; CBR, cerebrosides; DGDG, digalactosyl diglycerides; DGMG, digalactosyl monoglycerides; MGDG, monogalactosyl diglycerides; MGMG, monogalactosyl monoglycerides; NAPE, N-acyl-phosphatidyl ethanolamine; NALPE, N-acyl-lysophosphatidyl ethanolamine; PC, phosphatidyl choline; LPC, lysophosphatidyl choline

Table S4. Concentration of lipids from wheat dough after mixing (7 min) without proofing as affected by the addition of the lipase Lipopan Xtra and the solvent used for lipid extraction[a].

Lipid class[b]	Concentration [mg/kg dry matter]			
	Dough lipids with lipase added [mg/kg flour]			
	0	90	130	170
	Lipid extraction using 2-propanol (75 °C)			
TG	3035±222	2799±361	3068±110	2305±180
FFA	957±110	912±121	1096± 44	986± 35
DG	258± 54	228± 31	200± 14	324± 30
MG	121± 25	96± 4	87± 7	87± 29
ASG	72± 6	75± 5	72± 13	68± 11
SG	29± 14	13± 4	17± 1	22± 10
CBR	161± 13	201± 28	180± 10	174± 20
DGDG	1463±304	1372± 47	1546± 55	1337±116
DGMG	168 ± 62	160± 22	114± 28	159± 64
MGDG	434± 85	281± 13	209± 18	178± 27
MGMG	81± 14	149± 40	216± 98	232± 63
NAPE	493±116	383± 29	395± 82	380± 26
NALPE	384± 93	502± 61	563± 47	500± 75
PC	174± 79	315± 21	209± 58	164± 42
LPC	330± 97	565±136	639±250	535±350
	Lipid extraction using water-saturated n-butanol (20 °C)			
TG	4601±298	4864±406	4961±187	4960±282
FFA	1531±104	1912±385	1770±195	1896±245
DG	378± 11	285± 31	319± 22	324± 47
MG	159± 36	161± 12	191± 50	207± 42
ASG	100± 26	84± 25	97± 12	99± 27
SG	40± 5	43± 1	31± 1	30± 3
CBR	245± 67	234± 6	280± 17	292± 3
DGDG	2067±317	1714± 23	1894± 40	1857± 31
DGMG	96± 50	219± 32	230±120	225±116
MGDG	429± 53	281± 16	324± 53	346± 18
MGMG	290± 34	454± 7	498± 6	428± 18
NAPE	1055± 77	757±107	836± 11	720±120
NALPE	639±145	770± 92	824± 3	893± 9
PC	174± 48	203± 30	261±104	258± 27
LPC	500±232	844±390	595± 66	670±223

[a] mean values ± standard deviations of triplicate determinations
[b] TG, triglycerides; FFA, free fatty acids; DG, diglycerides; MG, monoglycerides; ASG, acylated sterol glucosides; SG, sterol glucosides; CBR, cerebrosides; DGDG, digalactosyl diglycerides; DGMG, digalactosyl monoglycerides; MGDG, monogalactosyl diglycerides; MGMG, monogalactosyl monoglycerides; NAPE, N-acyl-phosphatidyl ethanolamine; NALPE, N-acyl-lysophosphatidyl ethanolamine; PC, phosphatidyl choline; LPC, lysophosphatidyl choline

3.2 Reconstitution baking tests with defatted wheat flour are suitable to determine the functional effects of lipase-treated wheat lipids

A micro-scale reconstitution baking test using 2-propanol defatted flour (20°C) and isolated flour and dough lipids (WSB, 20 °C) was established by Monika Schaffarczyk to determine the functional effects of lipids isolated from lipase-treated wheat dough. After addition of lipase-treated dough lipids to defatted flour the bread volume increased up to 46%. Comparable results were obtained in a control experiment using non-defatted flour and lipases.

Proper selection of solvent and extraction temperature was of major importance to sustain the functionality of defatted flour. Dough and gluten from flour defatted with WSB (extracted at 20 °C) and 2-propanol (extracted at 75 °C) had inferior extensibility and loaf volume compared to control flour extracted with 2-propanol at 20 °C.

MixoLab data confirmed that flour extracted with 2-propanol at 20 °C was considered the most suitable defatted flour for reconstitution experiments compared to the two other extraction methods tested. Monika Schaffarczyk shared the responsibility for the performance and evaluation of the MixoLab experiments with her co-author.

One reason for this effect on the functional properties of defatted flour was the effect of defatting on the quantitative protein composition of wheat flour. Quantitation of gluten proteins showed that non-defatted control flour and the 2-propanol (20 °C) defatted flour had similar protein compositions, whereas defatting with 2-propanol at 75°C or WSB at 20 °C changed the protein composition by reducing the extractability of gliadins in 60% (v/v) ethanol, thus leading to increased glutenin content. Possible reasons were thiol-disulfide interchange reactions either caused by heat (2-propanol, 75 °C) or by the solvent WSB, which affected gluten proteins.

CLSM, performed by her co-authors, showed that regular, interconnected gluten structures were only present in dough from flour defatted with 2-propanol at 20 °C.

Additionally, Monika Schaffarczyk wrote the manuscript and revised it according to the comments of the reviewers.

3. Results

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Reconstitution baking tests with defatted wheat flour are suitable for determining the functional effects of lipase-treated wheat lipids

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ABSTRACT

A microscale reconstitution baking test, using wheat flour defatted with 2-propanol at 20 °C, was established to determine the functional effects of lipids isolated from lipase-treated wheat dough. Proper selection of solvent and extraction temperature was of major importance to maintain the functionality of defatted flour. Dough and gluten from flour defatted with water-saturated 1-butanol (WSB; extracted at 20 °C) and 2-propanol (extracted at 75 °C) had inferior extensibility and loaf volume compared to control flour extracted with 2-propanol at 20 °C. Quantitation of gluten proteins showed that defatting with WSB (20 °C) or 2-propanol (75 °C) decreased the gliadin and increased the glutenin content. Possible reasons were thiol-disulfide interchange reactions, caused either by heat (2-propanol, 75 °C) or by the solvent WSB, which affected gluten proteins. Confocal laser scanning microscopy showed that regular, interconnected gluten structures were only present in dough from flour defatted with 2-propanol at 20 °C.

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1. Introduction

Although lipids are present in wheat flour at considerably lower levels than starch or protein, they exhibit important functional properties in breadmaking. Selective modification of the wheat lipid composition by lipases drastically impacts the baking performance of the flour and the end-product quality (Moayedallaie, Mirzaei, & Paterson, 2010). Beside lipases, other exogenous enzymes are being used to improve the baking performance of wheat flours or to compensate for variations in quality of wheat flour. Currently available lipases for baking applications hydrolyze a number of lipid structures in flour (Gerits, Pareyt, & Delcour, 2013; Schaffarczyk, Oestdal, & Koehler, 2014) and lead to improved surface activity of endogenous lipids, thus resulting in significant increase in bread oven rise (Gerits, Pareyt, Masure, & Delcour, 2015) and specific volume (Gerits, Pareyt, & Delcour, 2014a). The understanding of lipase functionality is continuously being studied (Gerits, Pareyt, Decamps, & Delcour, 2014b), but is mostly based on the knowledge of the effects of added lipids in breadmaking. To investigate the contribution of flour components to baking performance, flour fractions can be added to native flour and the effects can be determined. For example, Selmair and Koehler (2009) analyzed the effects of individual glycolipid classes from commercial lecithins in breadmaking by means of microscale baking tests. It was shown that all isolated glycolipid classes had excellent baking performance. The disadvantage of such a method is that the composition of flour containing the additive, in particular the lipid content, is different from that of the control flour.

An important method for studying the contribution of lipid content or different lipid mixtures in breadmaking was fractionation and reconstitution (Pareyt, Finnie, Putseys, & Delcour, 2011). In this approach, flour is separated into fractions or components, which are then recombined to form reconstituted flour. With this method the reconstituted flour and the control flour contain the same amount of lipids. The extraction solvent is crucial because minimizing the effects of the solvent on flour functionality is a key for fractionation and reconstitution experiments. Especially for the complete extraction of the polar lipids, the proper selection of the extraction solvent and the extraction temperature is of

Abbreviations: AF, area fraction; ALGL, albumins/globulins; BI, branching index; BU, Brabender units; *C*, circularity; CLSM, confocal laser scanning microscopy; DF, Feret's diameter; EE, extensional energy; EX, extensibility; GLIA, gliadins; GLUT, glutenins; KLU, kilo-lipase-units; *P*, perimeter; RE, resistance to extension; RP-HPLC, reversed-phase high performance liquid chromatography; *S*, solidity; SD, standard deviation; TA, total area; WSB, water-saturated 1-butanol; ØA, average size; ΣP, particle count.

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major importance (Chung, Pomeranz, Finney, & Shogren, 1977; Chung, Pomeranz, Jacobs, & Howard, 1980). Water-saturated 1-butanol (WSB) is known to completely extract free and bound lipids of flour and dough. However, WSB-defatted flour is not suitable for studying the role of lipid mixtures because of its poor functional properties, possibly due to the formation of complexes between starch and WSB (Hoseney, Finney, Pomeranz, & Shogren, 1969; MacRitchie & Gras, 1973; Wieser, Antes, & Seilmeier, 1998).

To optimize lipase application in breadmaking, it is essential to understand functionality and the technological effects of reaction products at the molecular level. However, information about the functional effects of lipase-generated lipid classes is scarce. In particular, reconstitution baking tests would be a suitable approach to study the effects of lipases in breadmaking. To the best of our knowledge, no systematic fractionation–reconstitution studies have been done to unravel the functional effects of lipase-modified wheat lipids in breadmaking.

Therefore, the aim of this study was to develop a method for fractionation and reconstitution of wheat flour to investigate the functional effects of lipase-treated wheat lipids by means of microscale methods. The study was focussed on the production and characterization of fully functional defatted flour, suitable for recombination with lipase-treated dough lipids. The influence of the defatting method on the technological properties and the gluten network strength of doughs from defatted flours, as well as from recombined flours, should be determined by MixoLab tests and microscale extension tests, using the Kieffer rig. The results of such tests can be regarded as indirect quality parameters of the flour, which are correlated with the baking quality (Kieffer, Wieser, Henderson, & Graveland, 1998). The protein composition of flours should be quantitated by a modified Osborne fractionation to reveal changes in the protein compositions caused by treatment with different extraction solvents. The final aim was to visualize the gluten network structures of doughs from defatted and non-defatted flours by confocal scanning laser microscopy (CLSM). Once established, the new method would provide the ability to determine relationships between specific wheat lipid classes and their functional effects in wheat breadmaking.

2. Materials and methods

2.1. Materials

'Kolibri' flour, a commercial flour obtained from a mixture of wheat cultivars (Meneba, Rotterdam, The Netherlands, 2013 harvest, containing no additives), was characterized as follows: the moisture and ash contents of the flour were determined according to ICC-Standards 110/1 (ICC, 1976) and 104/1 (ICC, 1990), respectively. Nitrogen contents were determined by means of the method of Dumas on a TruSpec N nitrogen analyzer (Leco, Kirchheim, Germany). A conversion factor of 5.7 was used to calculate the crude protein content from the nitrogen content. Analytical characteristics of the flour were 14.3% moisture, 9.8% protein (dry mass), and 0.48% ash (dry mass).

Wheat starch (Sanostar) was obtained from Hermann Kröner GmbH (Ibbenbühren, Germany), wheat gluten (Amygluten 150) from Tereos Syral (Aalst, Belgium) and fresh baker's yeast from Wieninger GmbH (Passau, Germany). As in a previous study (Schaffarczyk et al., 2014), commercial enzyme granulate Lipopan F-BG (25.0 KLU/g) and commercial enzyme granulate Lipopan Xtra-BG (7.2 KLU/g) were from Novozymes A/S, Bagsvaerd, Denmark. Rhodamine B was from Sigma–Aldrich (Steinheim, Germany). All solvents used were of HPLC or LC–MS grade and from Sigma–Aldrich (Steinheim, Germany).

2.2. Lipid extraction

2.2.1. Lipid extraction from flour

Three different lipid extraction methods were compared regarding the functionality of the resulting defatted flour. Lipids were extracted from wheat flour, using WSB at room temperature (≈20 °C), 2-propanol at 75 °C, and 2-propanol at 20 °C, employing the procedure described by Schaffarczyk et al. (2014).

2.2.2. Lipid extraction from dough

Dough was prepared from 50 g of wheat flour as described recently (Schaffarczyk et al., 2014). The following ingredients were used: flour (50.2 g), water (29.9 ml), NaCl (1.0 g), and lipase (0–170 mg/kg flour; Lipopan F-BG or Lipopan Xtra-BG, Novozymes, Bagsvaerd, Denmark). The flour, NaCl, and lipase were premixed dry for 1 min in a Farinograph (50 g Z-blade mixer, Brabender, Duisburg, Germany) at 22 °C. Water (29.9 ml) was added within 25 s and mixing was continued until the optimum consistency of the dough was reached (550 Brabender units (BU) at 7 min). The dough was allowed to rest (20 min, 30 °C, water-saturated atmosphere). The dough was then reshaped on a dough rounder (Type 440, Brabender, Duisburg, Germany) for 10 cycles, and the resulting spherical dough piece was rolled (PTFE cylinder; diameter, 5 cm; length, 30 cm) to yield an oval dough piece of 5 mm thickness. The dough piece was folded twice to 1/4 of its original size and was reshaped on the dough rounder for 20 cycles. After resting (30 °C, water-saturated atmosphere, 38 min), the dough was frozen in liquid N_2, freeze-dried, and milled, using an ultracentrifugal mill ZM 200 (200 μm mesh size, Retsch, Haan, Germany), resulting in a powder with a mean particle size of 200 μm, which is larger than the particles of wheat flour (50–120 μm).

Lipids were extracted from the freeze-dried dough powder (about 44.5 g) by stirring with WSB (200 ml, 16 h, 20 °C). After centrifugation (5 min, 4 °C, 3550g), the supernatant was filtered (0.45 μm); the solvent was then evaporated to dryness under reduced pressure and stored under Ar atmosphere at −75 °C prior to further analysis.

2.3. Microbaking test (MBT)

2.3.1. General

The MBT, using 10 g of flour, was conducted as described by Schaffarczyk et al. (2014). To take dough losses during breadmaking into account, the loaf volumes were based on the dough weight (ml/g dough) before the second proofing session. The bread volume with modified lipid mixtures was expressed in relation to the respective mean value of the control breads in the individual test series, taking into account both climatic (temperature and relative humidity in the room) and technical fluctuations. All baking tests were done in triplicates.

2.3.2. MBT, using defatted flour

Recombined flour was prepared by adding different amounts of flour lipids, dough lipids or lipase-treated dough lipids to 10 g differently defatted flour (8.6 g dry mass) and blending with a mortar and a pestle. The recombined flour (10 g) was used for the MBT, as described recently (Schaffarczyk et al., 2014).

2.3.3. MBT using synthetic flour

Extracted untreated and lipase-treated dough lipids (0.15 g each) were each added to wheat starch (8.0 g) and wheat gluten (1.0 g) and blended with a mortar and a pestle. NaCl (0.2 g), glucose (0.2 g), and yeast (0.7 g) were added and premixed. After mixing for 1 min in a microfarinograph, gelatin-solution (4.6 ml, c = 43.5 g/l) and 0.54 ml water were added and the mixture was kneaded for 4 min to the dough optimum (550 BU). Dough

handling and preparation followed the same procedure as described above.

2.4. MixoLab tests

The standardized protocol AACCI 54-60.01 (AACCI, 2010) for complete flour characterization provided a torque [Nm]–time [min] diagram. Different dough types were prepared from native wheat flour or wheat flour defatted by different solvents, with and without addition of flour lipids extracted by WSB at 20 °C. Seven different dough samples were tested: wheat flour, wheat flour defatted by WSB, wheat flour defatted by WSB plus flour lipids, wheat flour defatted by 2-propanol (75 °C), wheat flour defatted by 2-propanol (75 °C) plus flour lipids, wheat flour defatted by iso-propanol (20 °C) and wheat flour defatted by 2-propanol (20 °C) plus flour lipids. All tests were run in duplicates.

The amounts of flour and water were calculated by the MixoLab programme from the flour moisture content (determined by NIR) in order to achieve a 75 g dough with a torque of 1.1 ± 0.05 Nm during the initial mixing of the dough. Native or defatted flours were added to the mixing bowl of the MixoLab (Villeneuve la Garenne, France). For samples with added flour lipid, the lipid was mixed into the flour with a spatula before the flour was transferred to the mixing bowl. Water was added automatically by the MixoLab, followed by initiation of the MixoLab programme (32 °C (0–8 min), 32–90 °C (8–23 min), 90 °C (23–30 min), 90–50 °C (30–40 min), 50 °C (40–45 min)).

2.5. Microscale extension tests of dough and gluten

Microscale extension tests with dough and gluten from 10 g of flour were carried out as reported by Köhler and Grosch (1999) with modifications displayed below. For dough rheology, non-defatted or defatted flour (10 g, 8.6 g of dry mass) and NaCl (0.2 g) were premixed dry in a microfarinograph for 1 min at 22 °C. Distilled water was added and mixing was continued until a maximum consistency of 550 BU was reached. After removal of the dough from the mixer, the dough was pressed into PTFE molds to give strands of 53 × 4 × 4 mm. After 15 min of resting, the strands were carefully taken from the PTFE molds and fixed at both ends into the Kieffer rig. Subsequently, the middle section of each strand was extended with a special hook on the Texture Analyzer TA-XT2 (Stable Microsystems) until it disrupted. Resistance to extension (RE, [N]), extensibility (EX, [mm]), and extensional energy (EE, [N × mm]) were recorded. Triplicate determinations were carried out, each providing 5 dough strands for testing.

For extension tests with wet gluten, wheat flour was mixed as described above. The dough was allowed to rest for 3 min at 22 °C and then washed for 10 min with a sodium chloride solution (0.4 M, 10 min) in a Glutomatic Type 2200 (Perten Instruments, Huddinge, Sweden) according to ICC-Standard No. 137/1 (ICC, 1994). The residue (gluten) was centrifuged for 10 min at 3060*g* and 22 °C, pressed into PTFE molds for 30 min, and extended, as described for wheat dough. Extension tests with re-hydrated gluten were carried out, using freeze-dried gluten (1.5 g) re-hydrated with NaCl-solution (5 ml; 2% w/v) for 5 min. Centrifugation, molding and analysis were done as described above. Triplicate determinations were carried out, each providing three gluten strands for testing (total of 9 determinations for each treatment).

2.6. CLSM, digital image processing and analysis

For the acquisition of CLSM micrographs, a Ti-U inverted research microscope with an e-C1plus confocal system (Nikon, Düsseldorf; Germany) and a 20× (N.A. = 0.75) objective was used. Dough was prepared as described by Döring, Nuber, Stukenborg, Jekle, and Becker (2015) in an adapted Glutomatic system 2000 (Perten, England): flour (4.3 g dry matter), NaCl (0.1 g), the determined amount of water (see Section 2.3.1), and aqueous rhodamine B solution (0.3 ml; c = 0.1 g/l) were mixed for 5 min. The protein phase was monitored as a fluorescence image (λ_{ex} = 543.5 nm, λ_{em} = 590/50 nm) with 2048 × 2048 pixel resolution (212 × 212 μm) in a constant z-position. Three different doughs were processed and analyzed from every experiment. Five micrographs of the control dough and 16 micrographs of the other samples were acquired at independent positions on the x/y-axis and further analyzed.

Image processing and analysis procedure followed the method of Jekle and Becker (2012). The micrographs were processed, using the image processing and analysis open source Java software ImageJ (version 1.48, National Institutes Health, Bethesda, MD, USA). After pre-processing by changing to an 8 bit gray-level, proteins were isolated from the background, using the threshold algorithm by Huang and Wang (1995). The binary micrographs were analyzed for the following protein features: particle count (ΣP, [–]), total area (TA, [μm²]), average size (ØA, [μm²]), area fraction (AF, [%]), perimeter (P, [μm]), circularity (C, [–]), Feret's diameter (DF, [μm]), solidity (S, [–], area of the particle divided by its convex area), and branching index (BI = P DF^{-1}, [–]).

2.7. Quantitation of protein fractions

The Osborne fractions albumins/globulins (ALGL), gliadins (GLIA), and glutenins (GLUT) of non-defatted and defatted flour were quantitated by an extraction/reversed-phase high performance liquid chromatography (RP-HPLC) method according to Wieser et al. (1998), using the modification of Thanhaeuser, Wieser, and Koehler (2014). Injection volumes were 20 μl for ALGL and GLUT and 10 μl for GLIA. For external calibration, reference gliadin (c = 2.5 mg/ml) of the Prolamin Working Group (van Eckert et al., 2006) was dissolved in 60% ethanol (v/v), injected (5, 10, 15 μl), and analyzed.

2.8. Statistical analysis

Data were statistically evaluated, using Microsoft Office Excel 2010 (Microsoft Corporation, Seattle, Washington, USA). One-way analysis of variance (ANOVA) of all quantitative data was calculated, using the software Sigma Plot 11.0 (Systat, San José, California, USA). The following values were used for rating of levels of significance: $p > 0.05$, not significant; $p < 0.05$, statistically significant; $p < 0.01$, significant; $p < 0.001$, highly significant.

3. Results and discussion

3.1. Microscale reconstitution baking test

To study functional effects of endogenous wheat lipids, modified wheat lipids, or wheat lipid fractions, reconstituted flour, consisting of defatted wheat flour and different wheat lipid mixtures, have to be used for breadmaking. In a recent study, we have shown that WSB at 20 °C is the preferred solvent for complete lipid isolation and have determined the optimal dosage of lipase (Schaffarczyk et al., 2014). The most suitable method for preparing the defatted flour for reconstitution remained to be developed. Four approaches were compared: flour defatted with WSB at 20 °C, with 2-propanol at 75 °C, and with 2-propanol at 20 °C. Finally, a fourth approach, using 'synthetic' flour composed of wheat starch, wheat gluten and gelatin, was used.

Initially, we used the MBT with native flour and lipase to set the target that a baking test with reconstituted flour (defatted flour plus lipids) had to meet. As shown in Fig. 1 significant volume increases in the MBT were obtained when the commercial lipases, Lipopan Xtra and Lipopan F, were added to native flour. This was in line with our recent results (Schaffarczyk et al., 2014). The volume increase correlated with the enzyme dosage until a plateau was reached. Lipopan F was found to reach the plateau at a lower level (70 mg of enzyme granulate/kg flour) compared to Lipopan Xtra (130 mg/kg flour). Further increase in enzyme dosage did not cause statistically significant differences in the obtained bread volumes.

In the first baking tests with reconstituted flour, the residue of the isolation of lipids with WSB at 20 °C was used as the defatted flour. WSB extracts more non-starch lipids from wheat flour at 20 °C than does any other solvent or solvent mixture (around 1.60% lipids) (Chung et al., 1980; MacRitchie & Gras, 1973; Morrison, Mann, & Coventry, 1975). However, this defatted flour was not suitable for breadmaking. In the second approach, a defatted flour, obtained by extraction with 2-propanol for 2 h at 75 °C, was used to maximize lipid extraction with minimal detrimental effects on flour functionality, as described earlier (Chung et al., 1977, 1980). Reconstitution MBTs were performed with this defatted flour (moisture content 7.25%, water absorption 67.3%, time to peak 9.5 min, and dough optimum at 543 BU) after addition of lipase-treated (30–170 mg/kg Lipopan F or Lipopan Xtra) and untreated dough lipids, also extracted with 2-propanol at 75 °C (lipid yield 1.35%/g flour). However, this system also did not appear to be suitable, because both additions of unmodified and lipase-modified lipids gave loaf volumes that were significantly smaller than the volumes obtained in the MBT with native flour (data not shown). Compared to WSB at 20 °C (lipid yield 1.6%), 2-propanol at 75 °C extracted less lipid (1.35%), and the WSB lipids contained more polar lipids than did the 2-propanol (75 °C) lipids (Schaffarczyk et al., 2014). To exclude a possible negative effect of the lower content of polar lipids in the lipids obtained with 2-propanol (75 °C) versus the lipids isolated with WSB at 20 °C on the bread volume of reconstituted breads, WSB-extracted lipids and 2-propanol-defatted flour (75 °C) were also recombined. However, there was no significant difference between untreated and lipase-treated lipids. This indicated that defatting with 2-propanol at 75 °C appeared to have a negative effect on the functionality of the defatted flour.

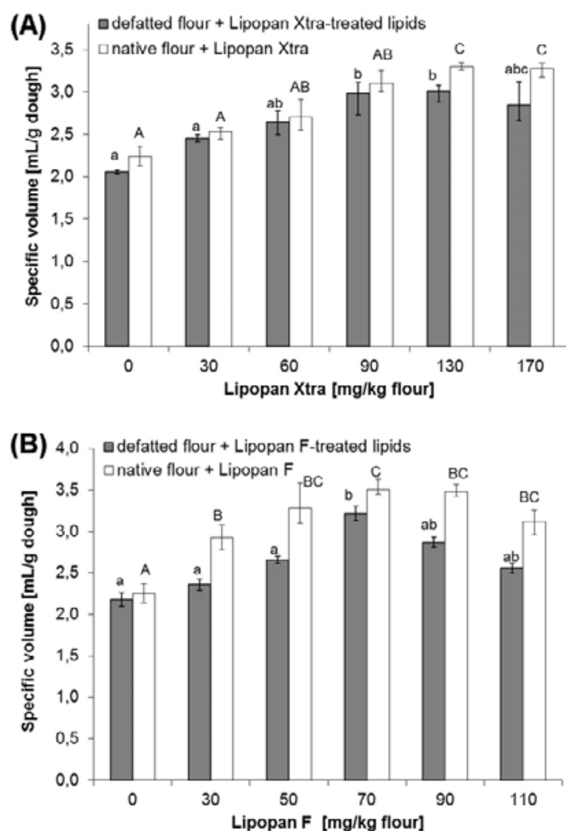

Fig. 1. Microbaking test with native and reconstituted flours. Specific volume of the bread as affected by increasing concentrations of lipase (native flour) and by addition of dough lipids modified with increasing concentrations of lipase (defatted flour). Reconstituted flours were recombined by mixing 9.88 g of 2-propanol-defatted flour (20 °C) and 0.12 g of WSB-extracted (20 °C) lipids. Commercial lipase samples: (A), Lipopan Xtra; (B), Lipopan F. Error bars represent mean standard deviations of triplicate determinations. Mean values associated with different letters are significantly different (one-way ANOVA, Tukey test, $p \leq 0.05$).

In addition, synthetic flour, according to Hartmann and Koehler (2008), was used for reconstitution experiments with some modifications. Beside wheat starch and wheat gluten, gelatin was used as a substitute for albumins and globulins and for compounds which increase the viscosity of the dough liquor. The water absorption was determined and adjusted to a dough consistency of 550 BU at 22 °C. Untreated, or lipase-treated dough lipids isolated with WSB at 20 °C, were added. Similar results were obtained as when using 2-propanol-defatted flour at 75 °C, with no significant change in bread volume effected by the different lipid mixtures. Therefore, the approach, using synthetic flour, was also not suitable for demonstrating the improving effect of lipase-treated lipids in wheat breadmaking.

Finally, breads were baked, using a defatted flour obtained by extracting wheat flour with 2-propanol for 2 h at 20 °C, to which flour or dough lipids extracted with WSB at 20 °C were added. As shown in Fig. 1, this system provided nearly the same functional properties as those obtained with native flour. The specific volume of the bread from native flour (2.15 ml/g dough) was not significantly different from the specific volumes of the reconstituted breads with flour lipids or dough lipids (2.14 ml/g dough or 2.06 ml/g dough), respectively. As the lipid yield with 2-propanol at 20 °C was 1.2%, compared to 1.6% with WSB at 20 °C (1.6%), 120 mg of WSB-extracted lipids (20 °C) were added back in the MBT, corresponding to the amount of lipids obtained from 10 g of flour. When lipids modified by different concentrations of the two lipases were added to the defatted flour, dose-dependent increases in loaf volume of up to 46% (Lipopan Xtra; 130 mg/kg flour) and 47% (Lipopan F; 70 mg/kg flour) were obtained. These bread volume increases were comparable to the experiments with native flour (volume increases of 47% or 56%, respectively) (Fig. 1). In summary, only the reconstitution MBT using defatted flour obtained by extraction with 2-propanol at 20 °C was suitable for determining the functionality of wheat lipids in breadmaking. Baking properties indicated that 2-propanol had no adverse effect on wheat flour components and only minor effects on gassing power and loaf volume. These data showed that, at an extraction temperature of 20 °C, lipids were extracted without irreversibly affecting the breadmaking performance of the flour, in contrast to the extraction temperature of 75 °C. In addition, proper selection of solvent for extracting lipids from flour is of major importance (Chung et al., 1980). As we wanted to examine the functional effects of lipase-treated lipids, which differed mainly in the polar lipid portion, extraction of the lipid fraction by WSB at 20 °C was chosen as described recently (Schaffarczyk et al., 2014).

3.2. MixoLab tests

The reconstitution MBT showed 2-propanol-defatted flour at 20 °C to have baking performance close to that of native flour, while flour defatted with either WSB at 20 °C or 2-propanol at 75 °C were found to have inferior functional properties. To study the different functionalities, flours defatted by the three protocols, with and without addition of flour lipids, were evaluated by MixoLab testing to characterize rheological behavior during mixing and heating of dough made from flour and water. The MixoLab test provides torque [Nm] of the dough as a function of time. Typical MixoLab curves are shown in Fig. 2. Flour defatted by 2-propanol at 20 °C was shown to have properties similar to those of native flour, especially when flour lipids were added. The difference between 2-propanol-defatted flour at 20 °C and native flour was mainly found during mixing and dough formation where the defatted flour showed slower build-up of torque. This could indicate weakening of the gluten network and a certain degree of difference in baking performance must be expected after extraction and subsequent drying of the defatted flour. MixoLab data for flour defatted by the two other defatting methods used showed substantially different MixoLab curves compared to native flour, confirming these two methods to be less suitable for reconstitution MBTs. For example, flour defatted with WSB (20 °C) and with 2-propanol (75 °C) showed a substantial increase in the value of the enzymatic degradation speed compared to the native flour. This was calculated by subtracting the gelatinization value (C3) from the hot gel stability value (C4). Increase in enzymatic degradation speed means increased loss of torque of the dough at high temperature and reflects lower dough stability during heat treatment. After addition of lipids to flour, defatted with 2-propanol at 20 °C, the enzymatic degradation speed value was similar to that of native flour. In contrast, recombined flour, after extraction with 2-propanol at 75 °C, showed a lower enzymatic degradation speed compared to the native flour. This decrease may be explained by a slightly different localization of the lipids when added to defatted flour compared to reference flour. Thus, flour extracted with 2-propanol at 20 °C was considered the most suitable defatted flour for reconstitution experiments when compared to the two other extraction methods tested.

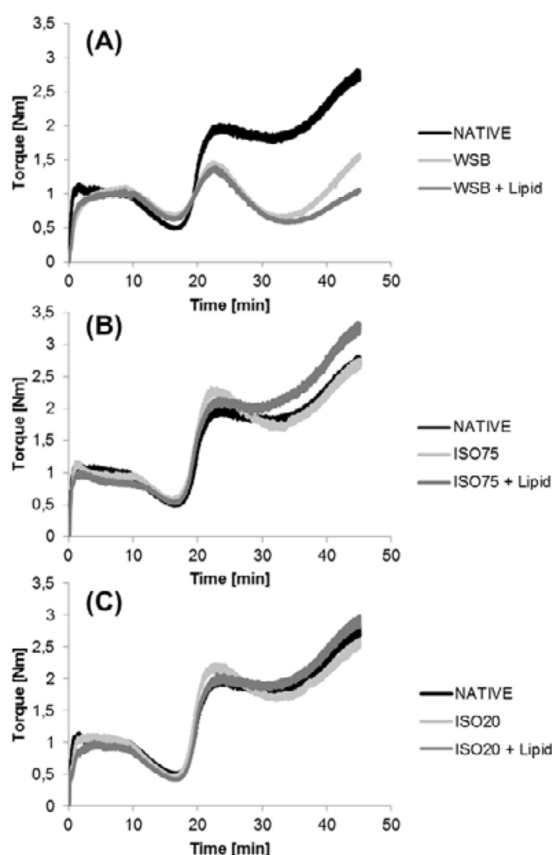

Fig. 2. MixoLab curves of native and defatted wheat flours, with and without addition of flour lipids extracted with WSB (20 °C). (A) Native flour (NATIVE), flour defatted with WSB at 20 °C (WSB), and reconstituted flour (WSB + lipid). (B) Native flour (NATIVE), flour defatted with 2-propanol at 75 °C (ISO75), and reconstituted flour (ISO75 + lipid). (C) Native flour (NATIVE), flour defatted with 2-propanol at 20 °C (ISO20), and reconstituted flour (ISO20 + lipid).

3.3. Microscale extension tests of dough and gluten

To further examine how the different extraction methods affected flour and gluten functionality, microscale extension tests with dough and gluten were carried out. Fig. 3 demonstrates the effects of defatting on dough rheology. It is obvious, that both EX and RE and, consequently, also the extensional energy (EE) of dough from flour defatted with WSB (20 °C) and 2-propanol (75 °C) were inferior to values in the non-defatted control flour. The total EE strongly correlates with the bread volume (Nash et al., 2006). The higher the value for the EE, the higher will the bread volume increase be. This was confirmed by the results obtained with dough of the defatted flours. Flours defatted with WSB (20 °C) and 2-propanol (75 °C), with weak baking performance, had the lowest EE. In contrast, dough from 2-propanol (20 °C)-defatted flour was closer to the control. This corresponded to its good baking performance.

The data from the microscale extension tests with gluten are shown in Supplementary Table 1. Both wet gluten and rehydrated freeze-dried gluten were studied. Wet gluten was isolated by washing dough with NaCl solution, using a Glutomatic. However, no residue (gluten) was recovered from the dough made from flour defatted with 2-propanol (75 °C) and WSB (20 °C). It can be assumed that defatting of flour under these conditions significantly affected the formation of a gluten network. Extension tests with wet gluten from non-defatted flour and 2-propanol-defatted flour (20 °C) showed no significant difference, which was in

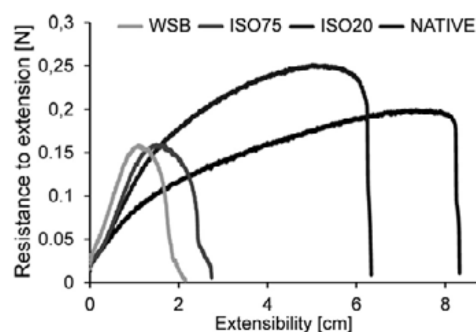

Fig. 3. Microscale extension tests with doughs from 10 g of native and defatted wheat flours. NATIVE, native flour; WSB, flour defatted with WSB at 20 °C; ISO75, flour defatted with 2-propanol at 75 °C; ISO20, flour defatted with 2-propanol at 20 °C.

agreement with the results obtained for extension tests of dough. However, the extensional behavior of re-hydrated gluten from defatted flour (2-propanol, 20 °C) was significantly different from that of the control because it exhibited significantly higher RE and lower EX. Papantoniou, Hammond, Scriven, Gordon, and Schofield (2004) described the effect of lipids on the water uptake of gluten. Lipid-containing gluten has a lower water uptake than has defatted gluten because lipids interact with amino acids side chains of the proteins and decrease the affinity of proteins for water. Defatted gluten is not protected from water, and access of water to the gluten proteins is not restricted. Preparation of wet gluten involves a washing step with excess water allowing unrestricted access of water to the proteins. In contrast, a fixed and possibly limiting amount of water (5 ml/1.5 g gluten) was added to freeze-dried gluten, whereas this amount of water was likely to sufficiently hydrate non-defatted dry gluten. Thus, comparable amounts of water would be expected in wet and re-hydrated gluten from non-defatted flour, whereas wet gluten from defatted flour would contain more water than would its re-hydrated counterpart. Given the function of water as a plasticizer in gluten, this could explain the increase of gluten strength, i.e. increased RE and decreased EE, in the case of re-hydrated, defatted gluten.

3.4. CLSM visualization of the protein microstructure

Visual analysis using CLSM provides information of the dough microstructure and enables the visualization of the developed protein network. Fig. 4 shows optical sections of wheat dough made from flour defatted with different extraction solvents. Protein (black) was stained with rhodamine B. Micrograph A of the control dough (non-defatted flour) exhibits a uniformly distributed protein network. The integrity of the gluten network structure is given by interactions between gluten strands and films (Jekle & Becker, 2011). The white holes in the continuous network indicate embedded starch granules, whereas a black ring around a white hole signals entrapped air bubbles. Micrograph B (dough from flour defatted with 2-propanol at 20 °C) shows more interconnected strains of protein and an intensive gluten network formation over the analyzed area. Because of this highly developed protein network, the RE was higher and the EX was lower compared to the control dough (see Fig. 3). Papantoniou et al. (2004) explained the more developed gluten network structure in dough from defatted flour by improved hydration as compared to dough from non-defatted flours. Dough made from flour defatted with 2-propanol (75 °C) (micrograph C) and WSB (20 °C) (micrograph D) showed no protein network formation. Precipitated protein particles were detected, which were scattered and clustered but not interconnected. It appeared that the defatting methods had a disaggregating effect on gluten proteins caused either by a combination of solvent and heat (2-propanol, 75 °C) or by the solvent (WSB, 20 °C). The fact that the protein particles were not interconnected explains why no wet gluten for extension tests could be isolated by washing the dough (see Section 3.3).

The micrographs were quantitatively evaluated by image analysis (Jekle & Becker, 2012). This revealed an increase of the area fraction (AF) for 2-propanol-defatted flour (20 °C) and a decrease for defatted flour at 75 °C or with WSB at 20 °C compared to the control flour (Fig. 5A). The AF represents the ratio of the area of the protein to the whole analyzed area. A denser or more spread protein network corresponds to a higher AF. Flour defatted with

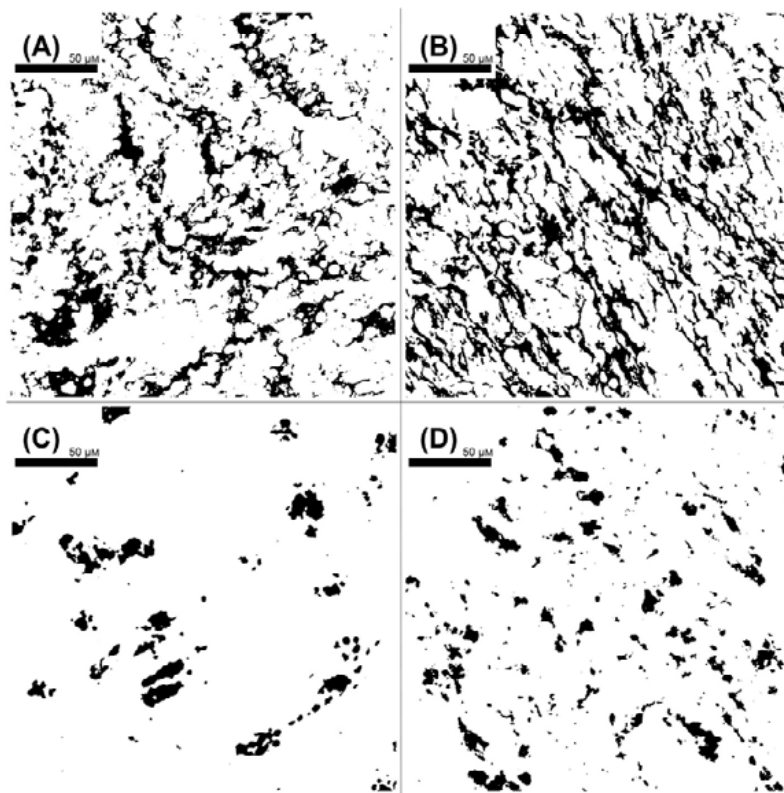

Fig. 4. Binary CLSM micrographs of doughs from native and defatted wheat flours. (A) Native flour. (B) Flour defatted with 2-propanol at 20 °C. (C) Flour defatted with 2-propanol at 75 °C. (D) Flour defatted with WSB at 20 °C. Proteins are displayed in black. Each micrograph has a size of 212 × 212 µm. The scale bar represents 50 µm.

M. Schaffarczyk et al. / Food Chemistry 200 (2016) 175–182

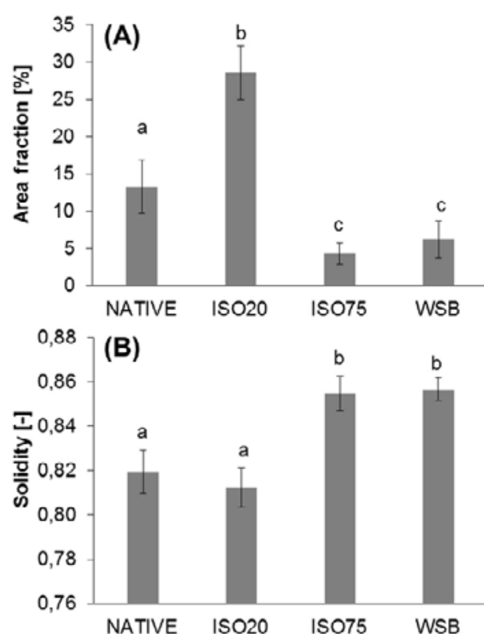

Fig. 5. Protein microstructure values of wheat doughs from native and defatted wheat flours obtained by image analyses of CLSM micrographs. NATIVE, native flour; ISO20; flour defatted with 2-propanol at 20 °C; ISO75, flour defatted with 2-propanol at 75 °C; WSB, flour defatted with WSB at 20 °C. (A) Area fraction (ratio of the area of the protein to the whole analyzed area). (B) Solidity (area of the particle divided by its convex area). Error bars represent mean standard deviations ($n = 5$ or $n = 16$). Mean values associated with different letters are significantly different (one-way ANOVA, Tukey test, $p \leqslant 0.05$).

2-propanol at 20 °C had a more spacious and better-connected protein network. The AFs of 2-propanol (75 °C) and WSB (20 °C) defatted flour showed a strong separation and compression/exclusion of the protein particles on a microscopic level. It seems that the gluten particles could not be stretched and elongated by the mechanical energy during kneading. According to Jekle and Becker (2012) this microstructure would correspond to dough with very low elasticity and firmness, and this corresponds to the results of the dough extension tests (Fig. 3). The solidity (*S*) is the area of the protein particle divided by the imaginary convex hull around it. A *S*-value of 1.0 means that a particle is solid and has no concave edges and, thus, has less connection area to other particles or the surrounding matrix. Therefore, this value can be also used for a morphological description. Doughs made of defatted flour with 2-propanol (75 °C) or WSB (20 °C) had a high *S*-value and could be interpreted as containing quite solid protein particles (Fig. 5B). In contrast, the doughs from the control and 2-propanol-defatted (20 °C) flours had lower and similar *S*-values and, thus, contained protein particles with a more branched and irregular surface. Thus, the proteins of 2-propanol-defatted (20 °C) flour formed chemical bonds, e.g. hydrogen, ionic, hydrophobic and covalent bonds, similar to the native ones, leading to a physically cross-linked network. This microscopic feature would enable interactions and loops on a microscopic scale in line with the loop and train model of gluten proteins of Belton (1999). This morphology has been postulated to lead to the physical material properties of elasticity and firmness (Jekle & Becker, 2015). The branching index (BI) is a further morphological measure for interactions. The expanded and widely spread gluten network in dough from flour defatted with 2-propanol at 20 °C resulted in a significantly higher BI than in dough from flour defatted with WSB or 2-propanol at 75 °C (Supplementary Table 2). In dough samples, the microscopic feature BI is correlated with values obtained by oscillation tests in a rheometer (Jekle & Becker, 2012). A high BI corresponds to high firmness and a high relative elastic part. In addition, Jekle and Becker (2012) described a correlation between BI and the RE, which was confirmed by our results: Dough from flour defatted with 2-propanol at 20 °C had the highest BI (CLSM) and the highest RE in the microscale extension test on the Kieffer rig (see Section 3.3).

3.5. Quantitation of protein fractions

The flour proteins of non-defatted and the three defatted flours were analyzed by means of an extraction/RP-HPLC method according to Wieser et al. (1998). Detection was based on the UV absorbance at 210 nm because, under these conditions, the HPLC absorbance areas are highly correlated with the amount of protein. The method was externally calibrated by analyzing standard solutions of PWG-gliadin. The results of these analyses are summarized in Table 1. The non-defatted control flour and the 2-propanol (20 °C) defatted flour had similar protein compositions. No significant differences in the concentrations of ω5-, ω1,2-, α- and γ-gliadins and glutenin-bound ω-gliadins (ωb-gliadins), high- and low-molecular-weight subunits of glutenin were present. In contrast, flours defatted with 2-propanol (75 °C) and WSB (20 °C) showed different protein compositions, which resembled each other. The extractability of gliadins with 60% ethanol was lower than in the control, leading to a considerably lower gliadin/glutenin ratio. A possible reason might be thiol-disulfide interchange

Table 1
Crude protein (CP) content determined by the method of Dumas and content of albumins/globulins (ALGL), gliadins (GLIA), glutenins (GLUT), gluten (= GLIA + GLUT), sum of extractable protein (Σ) and protein types of GLIA and GLUT determined by modified Osborne-fractionation of native wheat flour (NATIVE), wheat flour defatted with 2-propanol at 20 °C (ISO20), 2-propanol at 75 °C (ISO75), and water-saturated 1-butanol at 20 °C (WSB).

Sample	CP [mg/g flour][a]	ALGL [mg/g flour][a]	GLIA [mg/g flour][a]	GLUT [mg/g flour][a]	Gluten[b] [mg/g flour]	Σ [mg/g flour]	GLIA/GLUT
NATIVE	98.6 ± 1.4	13.7 ± 0.2	54.2 ± 3.5	28.6 ± 2.4	82.8	96.5	1.90
ISO20	101.9 ± 0.6	14.1 ± 0.2	56.2 ± 2.8	29.3 ± 1.1	85.5	99.6	1.92
ISO75	101.8 ± 0.2	12.5 ± 0.3	49.8 ± 0.7	34.5 ± 0.8	84.3	96.8	1.44
WSB	98.8 ± 0.5	9.0 ± 0.1	46.0 ± 1.9	33.9 ± 1.2	79.9	88.9	1.36

Sample	ω5-GLIA [mg/g flour][a]	ω1,2-GLIA [mg/g flour][a]	α-GLIA [mg/g flour][a]	γ-GLIA [mg/g flour][a]	ωb-GLIA [mg/g flour][a]	HMW-GS[c] [mg/g flour][a]	LMW-GS[c] [mg/g flour][a]
NATIVE	3.2 ± 0.1	4.5 ± 0.2	26.5 ± 1.4	20.0 ± 1.6	1.3 ± 0.2	8.8 ± 0.4	18.5 ± 1.7
ISO20	3.6 ± 0.1	4.9 ± 0.2	28.2 ± 1.4	19.5 ± 1.2	1.3 ± 0.1	9.0 ± 0.2	19.0 ± 0.8
ISO75	3.4 ± 0.1	4.7 ± 0.1	24.7 ± 0.3	17.0 ± 0.3	1.3 ± 0.0	10.0 ± 0.2	23.2 ± 0.6
WSB	3.2 ± 0.1	4.5 ± 0.2	23.0 ± 0.9	15.3 ± 0.7	1.3 ± 0.0	9.5 ± 0.4	23.1 ± 0.8

[a] Mean value of triplicate determinations ± standard deviation, based on fresh weight.
[b] Sum of GLIA + GLUT.
[c] HMW-GS, high-molecular-weight glutenin subunits; LMW-GS, low-molecular-weight glutenin subunits.

reactions between gluten protein types caused either by heat (2-propanol, 75 °C) or by the disaggregating effect of WSB (20 °C) (Hoseney et al., 1969). In agreement with Wieser (1998), cysteine-free ω-gliadins were less affected than were α- and γ-gliadins. The latter became covalently bound to the polymeric glutenins and, thus, were only extractable under reducing conditions (= glutenin fraction). The solvent WSB (20 °C) had an additional effect. Chloroform–methanol (CM-) extractable proteins were already extracted during defatting of flour with WSB, resulting in a reduced content of the albumin/globulin fraction. The quantitation of the protein fractions, as affected by the defatting method, showed moderate differences, but in our view these changes in the protein composition could not explain that 2-propanol (75 °C)- and WSB- (20 °C) defatted flour were not capable of forming a functional gluten network.

4. Conclusion

Wheat flour defatted with 2-propanol at 20 °C and WSB-isolated wheat lipids are suitable for reconstitution experiments. This system results in the same functionality as native wheat flour and allows study of the functional effects of any type of lipid in breadmaking, including flour and dough lipids from wheat, lipase-modified wheat lipids, unmodified or modified wheat lipid fractions, individual lipid classes, and even non-wheat lipids. Other defatting methods, using 2-propanol under heating (75 °C) or WSB at 20 °C, lead to a partial or complete loss of flour functionality in baking applications. Beside the extraction solvent, the temperature also affects the flour properties. 2-Propanol at 75 °C has a disaggregating effect on gluten proteins and changes the protein functionality, while ambient temperature provides native-like proteins. All in all, the established microscale reconstitution baking test can be used to determine structure–function relationships of specific wheat lipid classes in breadmaking. This should allow unravelling of the specific contribution of lipase reaction products to the improved baking performance of wheat dough after addition of baking lipases. Based on the system established in this study, it will be possible to identify optimal lipid mixtures for breadmaking and to gain insight of the mechanism of action of baking lipases. Such investigations are currently under way.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.01.010.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1

Microscale extension test with dough and gluten from 10 g of native wheat flour (NATIVE), wheat flour defatted with 2-propanol at 20 °C (ISO20), 2-propanol at 75 °C (ISO75), and water-saturated 1-butanol at 20 °C (WSB). Maximum resistance to extension (RE), extensibility (EX), and extensional energy (EE).

Dough/gluten sample	RE[a] [N]	EX[a] [mm]	EE[a] [N × mm]
		Dough	
NATIVE	0.20 ± 0.00	73.5 ± 1.2	10.7 ± 0.4
ISO20	0.24 ± 0.03	52.0 ± 2.1	9.0 ± 0.7
ISO75	0.16 ± 0.01	15.7 ± 0.6	1.4 ± 0.1
WSB	0.17 ± 0.00	12.8 ± 0.8	1.3 ± 0.1
		Wet gluten	
NATIVE	0.74 ± 0.02	112.6 ± 1.2	42.9 ± 2.5
ISO20	0.73 ± 0.04	115.3 ± 6.1	44.6 ± 0.8
		Freeze-dried and re-hydrated gluten	
NATIVE	0.97 ± 0.07	145.55 ± 6.3	74.43 ± 7.5
ISO20	1.64 ± 0.08	90.93 ± 1.8	74.90 ± 5.7

[a] Mean value of triplicate determinations ± standard deviation

Supplementary Table 2

Parameters related to protein properties obtained after image processing of CLSM micrographs of doughs from native wheat flour (NATIVE), wheat flour defatted with 2-propanol at 20 °C (ISO20), 2-propanol at 75 °C (ISO75), and water-saturated 1-butanol at 20 °C (WSB).

Protein feature[a]	Sample			
	NATIVE[b]	ISO20[c]	ISO75[c]	WSB[c]
ΣP [-]	780 ± 280	753 ± 117	98 ± 21	321 ± 116
TA [µm²]	558,857 ± 150,853	1,120,620 ± 284,191	182,931 ± 59,987	262,337 ± 104,090
ØA [µm²]	773 ± 243	1,531 ±493	1,854 ± 366	805 ± 113
AF [%]	13.3 ± 3.6	28.6 ± 3.6	4.3 ± 1.4	6.3 ± 2.5
P [µm]	105.4 ± 17.0	166.7 ± 35.2	143.1 ± 22.3	95.0 ± 8.1
C [-]	0.74 ± 0.04	0.70 ± 0.01	0.73 ± 0.02	0.76 ± 0.01
S [-]	0.82 ± 0.01	0.81 ± 0.01	0.85 ± 0.01	0.86 ± 0.01
DF [µm]	31.2 ± 5.8	39.2 ± 8.7	46.1 ± 6.6	31.6 ± 2.3
BI [-]	3.40 ± 0.23	4.31 ± 0.44	3.10 ± 0.16	3.01 ± 0.05

[a] ΣP, particle count; TA, total area; ØA, average size; AF, area fraction; P, perimeter; C, circularity; S, solidity; DF, Feret's diameter; BI, branching index

[b] Mean value ± standard deviation (n = 5)

[c] Mean value ± standard deviation (n = 16)

3.3 Relationships between lipase-treated wheat lipid classes and their functional effects in wheat breadmaking

The activity-guided lipid fractionation of lipase-treated lipids showed that polar lipase-reaction products were responsible for the positive functional effects of lipids in wheat breadmaking. The bread volume increase was even higher for polar lipase-treated lipids than for polar untreated lipids.

Monika Schaffarczyk further fractionated the polar fractions of lipase-treated (Lipopan Xtra 130 mg/kg flour) and untreated dough lipids by column chromatography providing six and eight sub-fractions enriched in specific lipid classes, respectively. The results allowed the conclusion that an optimal baking lipase preferably hydrolyses MGDG and NAPE, but has only moderate activity toward DGDG. Synergistic effects of DGDG and their products DGMG (molar proportion 1:1) provided even a greater bread volume increase than DGDG alone. These synergistic effects between lipids are probably based on the indirect mechanism of lipids on the stabilization of the gas cell interface of dough. DGMG only have an improving effect in combination with other lipids promoting the lamellar or/and hexagonal phase I. This denotes the importance of the optimum lipid composition and the breakdown of lipids to obtain an increase of the bread volume.

Quantitation of the lipid classes showed that overproofing of lipase-containing dough caused a decrease of the bread volume due to extensive lipid hydrolysis resulting in a decrease of lipids forming a lamellar mesophase thus providing inferior stabilization of gas cells.

Reconstitution baking (defatted flour plus lipid or lipid fraction added) partly provided contradictory results. 100% polar lipase-treated lipids were less active than untreated dough lipids and flour lipids. Combinations of non-polar and polar lipase-treated lipids were even more active than the polar ones alone. We assume that the polar reaction products of baking lipases need the presence of unmodified base lipids or non-polar lipids to provide the maximal positive functional effects. Synergistic effects of different lipid classes might play a key role in the mechanism of baking lipases.

Monika Schaffarczyk partly designed the experiments, she performed the experiments, collected and evaluated the data, wrote the manuscript and revised it according to the comments of the reviewers.

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Relationships between lipase-treated wheat lipid classes and their functional effects in wheat breadmaking

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Fractionation of lipase-treated wheat lipids showed that polar lipase-reaction products were responsible for their positive functional effects in breadmaking. The results allowed the conclusion that an optimal baking lipase preferably hydrolyzes monogalactosyl diglycerides and N-acyl phosphatidyl ethanolamine, but has only moderate activity towards digalactosyl diglycerides. Synergistic effects of digalactosyl diglycerides and their products digalactosyl monoglycerides (molar proportion 1:1) provide even a greater bread volume increase. Reconstitution baking (defatted flour plus lipid or lipid fraction added) partly provided contradictory results. 100% polar lipase-treated lipids were less active than untreated polar dough lipids plus polar flour lipids. Combinations of non-polar and polar lipase-treated lipids were even more active than the polar ones alone. It appears that the polar reaction products of baking lipases need the presence of unmodified lipids or non-polar lipids to provide optimal functional effects. The present data suggests that synergistic effects between different lipid classes might play a key role in the mechanism of action of baking lipases.

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1. Introduction

Despite the fact that lipids represent a minor fraction of wheat flour, the composition and structure of wheat flour lipids strongly influence the end-use quality of the bread. Lipases have been applied over the past two decades in the process of breadmaking to improve dough processing and the bread quality (Poulsen et al., 1998; Primo-Martín et al., 2008; Moayedallaie et al., 2010; Colakoglu and Özkaya, 2012; Gerits et al., 2014a). Lipases can be used to partially or completely replace synthetic emulsifiers like DATEM which are extensively used in the bread industry to provide increase in bread oven rise and specific volume (Pareyt et al., 2011).

The mechanisms underlying the functional effects of lipases are linked to the hydrolysis of one or more fatty acid moieties from non-polar and polar lipids to generate more polar lipids leading to improved surface activity and, thus, stronger functional effects of these endogenous lipids. Analysis of lipase-treated wheat lipids revealed that the hydrolysis of galactolipids and phospholipids are most important to improve the final the bread volume (Gerits et al., 2013, 2014b; Schaffarczyk et al., 2014). De Stefanis and Ponte (1976) postulated that glycolipids are more active in baking than phospholipids. The non-polar lipids, especially the triglycerides (TG) and the generated free fatty acids (FFA), both remain in the free lipid fraction and are extractable with hexane. The polar lipids belong to the bound fraction, which is entrapped in the gluten network. Gerits et al. (2015) showed that dough liquor recovered from lipase-treated dough contained higher lipid levels than that of dough liquor recovered from control dough. The liquid film as part of dough liquor is considered the medium for incorporation and growth of gas cells in dough (MacRitchie, 1976; Primo Martin et al., 2006; Salt et al., 2006). This means that the higher the concentration of surface-active polar lipids in the dough liquor induced by lipase, the higher the impact on bread volume.

Abbreviations: ASG, acylated sterol glucosides; BU, Brabender units; DCM, dichloromethane; DG, diglycerides; DATEM, diacetyl tartaric acid esters of mono- and diglycerides; DGDG, digalactosyl diglycerides; DGMG, digalactosyl monoglycerides; dm, dry matter; FFA, free fatty acids; HPLC-ELSD, high performance liquid chromatography coupled with an evaporative light scattering detector; HPTLC, high performance thin layer chromatography; KLU, kilo lipase-units; LPC, lysophosphatidyl choline; MG, monoglycerides; MBT, micro-baking test; MGDG, monogalactosyl diglycerides; MGMG, monogalactosyl monoglycerides; NALPE, N-acyl-lysophosphatidyl ethanolamine; NAPE, N-acyl-phosphatidyl ethanolamine; PC, phosphatidyl choline; SD, standard deviation; SG, sterol glucosides; TG, triglycerides; WSB, water-saturated 1-butanol.

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Gerits et al. (2015) suggested that dough rheology is only impacted by the lipid composition readily available at the start of mixing and not by the lipase-reaction products after fermentation. Since dough rheology examined by Kieffer rig extension tests was not affected by lipases, the effect of lipases has to be caused by a direct stabilization mechanism of the liquid film surrounding the gas cells. The most probable mechanism of stabilization of the gas cells by surface-active lipids is the formation of condensed monolayers at the gas–liquid interface. These condensed monolayers generate substantial elastic restoring forces, which resist destabilization of liquid lamellae when changes in the interfacial areas occur (Sroan and MacRitchie, 2009). Lipids that give expanded monolayers are relatively compressible and lead to instability in the liquid lamellae. It was speculated that lipids forming a hexagonal phase I type phase, for example FFA, are able to emulsify the deleterious lipids thereby preventing their adsorption into the interface and helping in building of expanded monolayers.

It was shown that an overdose of lipase caused bread volume decrease (Gerits et al., 2014b; Schaffarczyk et al., 2014). Lipid analysis indicated extensive lipid hydrolysis caused by the high enzyme concentrations. This denotes the importance of the optimum lipid composition by selective breakdown of lipids to optimize bread volume increase. Moreover, lipases probably catalyze the transition of lipid classes promoting the hexagonal II mesophase to lipid classes promoting the lamellar and hexagonal I mesophases, which improve stabilization of gas cells. Overdosing of lipases decreases the level of lipid classes building condensed monolayers resulting in the destabilization of gas cells (Gerits et al., 2014b). Further, overdosing induces earlier termination of oven rise due to gas cell coalescence (Gerits et al., 2015).

Until now, it is not clear, whether and to what extent each endogenous lipid class should be hydrolyzed by a baking lipase to yield a lipid mixture providing the best gas cell stability. Altogether, in-depth understanding of lipase-mediated reaction products is missing and especially information regarding the functional effects of lipid classes generated by the action of lipases is still very scarce. None of the studies published so far used fractionation of lipid classes before and after lipase addition to show the effect of individual lipid classes on the baking performance. Against this background, the aim of this study was to determine the relationships between specific wheat lipid classes and their functional effects in the baked product. Different lipid mixtures and fractionated polar and non-polar fractions should be analyzed for their functional effects using a recently established micro-reconstitution baking test with 10 g 2-propanol defatted wheat flour (Schaffarczyk et al., 2016). To determine the relation between lipid mixtures and their functional effects in breadmaking, combinations of polar and non-polar lipids as well as of specific isolated wheat lipid classes were quantitated by high-performance liquid chromatography with evaporative light scattering detection (HPLC-ELSD).

2. Materials and methods

2.1. Wheat flour

'Kolibri' flour, a commercial flour obtained from a mixture of wheat cultivars (Meneba, Rotterdam, The Netherlands, 2014 harvest, containing no additives), was characterized as follows: The moisture and ash contents of the flour were determined according to ICC Standards 110/1 (ICC, 1976) and 104/1 (ICC, 1990), respectively. The protein content (N × 5.7) was determined by the Dumas method by means of a TruSpec N nitrogen analyzer (Leco, Kirchheim, Germany). Analytical characteristics of the flour were 13.5% moisture, 9.7% protein (dry matter (dm)), and 0.59% ash (dm). Further analytical and functional data of the flour are reported elsewhere (Schaffarczyk et al., 2016).

2.2. Chemicals and reagents

Fresh baker's yeast (*Saccharomyes cerevisiae;* standard yeast for breadmaking applications) was obtained from Wieninger GmbH (Passau, Germany). Lipid standards were as described in Schaffarczyk et al. (2014). Silica gel (0.063–0.200 mm) and concentrating zone-HPTLC plates (20 × 20 cm) coated with silica gel G 60 (20 × 5 cm) on glass were from VWR Merck (Darmstadt, Germany). The following lipase samples were used: Commercial enzyme granulates Lipopan F-BG (25.0 KLU/g) and Lipopan Xtra-BG (7.2 KLU/g), Novozymes A/S, Bagsvaerd, Denmark; commercial enzyme granulate Panamore Golden, DSM, Delft, The Netherlands; experimental sample enzyme granulate EL 2013 000 405, AB Enzymes, Darmstadt, Germany. All solvents used were HPLC or LC-MS grade and purchased from Sigma–Aldrich (Steinheim, Germany).

2.3. Defatting of flour

Flour (1000 g) and 2-propanol (2.5 L) were stirred for 1 h at 20 °C. After centrifugation (20 min, 4 °C, 3550 × *g*), the solvent was removed by filtration on a glass filter (porosity 3; pore size 16–40 μm). The flour was re-extracted twice (2 × 2.5 L, 30 min). The defatted flour was spread out on a flat glass tray and left to dry in a fume hood for one week.

2.4. Lipid extraction from flour

Flour lipids were extracted from flour using water-saturated 1-butanol (WSB) as described by Schaffarczyk et al. (2014).

2.5. Lipid extraction from dough

Dough was prepared by upscaling the recipe based on 10 g of flour described by Koehler and Grosch (1999). Flour (298.8 g), NaCl (6 g), and Lipopan Xtra (0 and 39 mg; 0 and 130 mg/kg flour) were mixed for 1 min in a Farinograph (300 g Z-blade mixer; Brabender, Duisburg, Germany) at 22 °C. Water (179.4 mL) was added within 25 s and mixing was continued until the optimum consistency of the dough (550 Brabender Units (BU) at 7 min) was reached. The dough was allowed to rest for 20 min at 30 °C in a water-saturated atmosphere. The dough was then reshaped on a dough rounder (Type 440; Brabender, Duisburg, Germany) for ten cycles and the resulting spherical dough piece was rolled (PTFE cylinder, diameter 5 cm, length 30 cm) to yield an oval dough piece of 5 mm thickness. The dough piece was folded twice to ¼ of its original size and reshaped on the dough rounder for 20 cycles. After proofing (30 °C, 38 min) the dough was frozen with liquid N_2, freeze-dried, and milled using an ultracentrifugal mill ZM 200 (200 μm mesh size, Retsch, Haan, Germany). The milled freeze-dried dough was stirred in WSB (1.3 L, 16 h, 20 °C) to extract lipids. After centrifugation for 20 min (3550 × g, 4 °C), the supernatant was filtered through a fluted filter paper. The solvent was evaporated to dryness under reduced pressure and stored under Ar atmosphere at −75 °C until further analysis.

2.6. Separation of non-polar and polar lipids by batch procedure

The silica gel (G 60, 0.040–0.063 mm) was activated as follows: Silica gel (200 g) was dried overnight at 105 °C and left to cool in a desiccator. After adding water (10 mL) the material was shaken for 20 min. Flour lipids, dough lipids or lipase-treated dough lipids (15 g) were dissolved in diethyl ether (100 mL) and added to activated silica gel (200 g). To extract the non-polar lipids, diethyl

ether/petrol ether (boiling range 40–60 °C) (350 mL; 90/10, v/v) was used as extraction solvent (Ponte and De Stefanis, 1969). This lipid-silica gel slurry was kept stirring for 15 min. After centrifugation for 10 min (3550 × g) the supernatant was filtered through a fluted filter paper. Six further extractions of the silica gel residue were made with diethyl ether/petrol ether (6 × 350 mL; 90/10, v/v) in the same manner as described above. To extract the remaining lipids from the silica gel, methanol/water (5 × 350 mL; 90/10, v/v) was used. The combined diethyl ether/petrol ether fractions and the combined methanol/water fractions were evaporated to dryness under reduced pressure and analyzed by HPLC-ELSD according to Schaffarczyk et al. (2014).

2.7. Fractionation of polar lipids by silica gel column chromatography

The silica gel (G 60, 0.040–0.063 mm) was activated as follows: Silica gel (400 g) was dried overnight at 105 °C and left to cool in a desiccator. After adding water (20 mL) the material was shaken for 20 min. The activated silica gel was dispersed in dichloromethane (DCM), poured into a glass column, and packed to a height of 38 cm (diameter 5 cm). Polar lipid fractions (isolation: see above) from Lipopan Xtra-treated (130 mg/kg flour) or untreated dough lipids were purified according to the modified procedure of Folch (1957) as described by Selmair and Koehler (2008). Purified polar lipids (5.0 g) were dissolved in DCM and applied to the column. The lipids were eluted with DCM (1 L), DCM/methanol (3 L; 95/5, v/v), DCM/methanol (2 L; 90/10, v/v), DCM/methanol (2 L; 85/15, v/v), DCM/methanol (2 L; 80/20, v/v), DCM/methanol (1 L; 70/30, v/v), DCM/methanol (1 L; 60/40, v/v), DCM/methanol (1 L; 50/50, v/v) and methanol (1 L). Fractions (250 mL) were collected by a fraction collector, evaporated, and examined by HPTLC as described by Selmair and Koehler (2009). Fractions enriched in the same lipid classes were combined and analyzed by HPLC-ELSD (Schaffarczyk et al., 2014).

2.8. Reconstitution micro-baking tests (MBT) with lipid mixtures

The MBT using 10 g of flour was conducted as described by Schaffarczyk et al. (2016). To take dough losses during breadmaking into account, the loaf volumes were based on the dough weight (mL/g dough) before the second proofing session. The bread volume obtained with modified lipid mixtures was expressed as percentage of the control bread in the individual test series, in order to take into account climatic (temperature and relative humidity in the lab) fluctuations, i.e. the breads were prepared in a controlled air-conditioned room (22 ± 2 °C, ≥50% rel. humidity) and bread volumes of samples were always compared to the average volume of the control samples in the particular baking trial. All baking tests were made in triplicates or otherwise stated. Different mixtures of extracted flour lipids as well as untreated or lipase-treated dough lipids (in total 120 mg or 0–120 mg) were added to defatted flour (9.50 g; 8.60 g dm) and blended with a mortar and a pestle (= recombined flour). Recombined flour, NaCl (0.2 g), sucrose (0.1 g), and baker's yeast (0.7 g) were premixed dry for 1 min in a microfarinograph (10 g Z-blade mixer, Brabender, Duisburg, Germany) at 22 °C. A L-threo-ascorbic acid solution (0.3 mL, 0.67 g/L) and water (amount determined according to ICC-Standard 115/1 (ICC, 1992)) were added within 25 s and mixing was continued until the optimum consistency of the dough (550 BU at 7 min) was reached. After removal from the mixer, the dough was fermented (20 min, 30 °C, water-saturated atmosphere). The dough was then reshaped on a dough rounder (custom made) for 10 cycles. The resulting dough ball passed through the rolls (3 mm roll-gap) of an AMPIA-pasta machine (model 150 mm - Deluxe, Marcato, Italy) to form an oval dough piece. This piece was folded twice to ¼ of its original size and reshaped on the dough rounder for 20 cycles. The dough was then proofed (30 °C, water-saturated atmosphere, 38 min) and baked (180 °C increasing to 250 °C within 10 min) on an automatic proofing-baking line (custom made). The volume of the bread was determined using a benchtop laser-based device (VolScan Profiler, Stable Micro Systems, Godalming, U.K.).

2.9. Kinetic experiments

Dough was prepared from wheat flour using the following ingredients: Flour (9.94 g), water (5.98 mL), NaCl (0.2 g), and lipases (Lipopan Xtra-BG, 130 mg/kg flour; Lipopan F-BG, 70 mg/kg flour; Panamore Golden, 70 mg/kg flour; EL 2013 000 45, 70 mg/kg flour, respectively). The flour, NaCl, and lipase were premixed dry for 1 min in a microfarinograph (10 g Z-blade mixer, Brabender, Duisburg, Germany) at 22 °C. Water was added within 25 s and mixing was continued until the optimum consistency of the dough (550 BU at 7 min) was reached. After proofing (30 °C, water-saturated atmosphere, 0–120 min), the dough was frozen in liquid N_2, freeze-dried, and milled using an ultracentrifugal mill ZM 200 (200 µm mesh size, Retsch, Haan, Germany). Lipids were extracted from the freeze-dried dough powder (about 9 g) by stirring with WSB (40 mL, 16 h, 20 °C). After centrifugation (10 min, 3550 × g), the supernatant was filtered (0.45 µm membrane filter), the solvent was evaporated to dryness under reduced pressure and analyzed by HPLC-ELSD (Schaffarczyk et al., 2014).

2.10. Statistical analysis

Data was statistically evaluated using Microsoft Office Excel 2010 (Microsoft Corporation, Seattle, Washington, USA). One-way analysis of variance (ANOVA) of all quantitative data was calculated using the software Sigma Plot 11.0 (Systat, San José, California, USA). The following values were used for rating of levels of significance: $p > 0.05$, not significant; $p < 0.05$, statistically significant.

3. Results and discussion

3.1. General outline of the study

In a recent study, we have established a method for fractionation and reconstitution of wheat flour to investigate the functional effects of lipase-treated wheat lipids by means of our micro-scale baking method using 10 g of flour (Schaffarczyk et al., 2016). In the present study, wheat flour defatted with 2-propanol at 20 °C and different WSB-extracted wheat lipids, such as flour lipids, dough lipids, lipase-treated dough lipids and fractions thereof were used for reconstitution experiments. Bread samples with untreated lipids and lipid fractions served as controls. The loaf volume was considered the most important parameter, while possible effects on bread staling were not studied. The work was divided into four parts. In the first part, WSB-extracted untreated or lipase-treated dough lipids were fractionated into a non-polar fraction and a polar fraction and subsequently were assayed for their functional effects in our micro-scale baking system. In the second part, polar lipid fractions were separated further by column chromatography to yield sub-fractions enriched in specific lipid classes and evaluated by micro-scale baking. Then, studies on synergistic effects of lipid classes were conducted to get insight into effects of lipid mixtures. For this purpose, the role of background lipids (extracted from native flour) in the reconstitution baking test and the absolute lipid concentration on functional effects were determined. Finally, kinetic studies using four different lipases were conducted to provide the incubation times that provided the most beneficial lipid

mixtures.

3.2. Functional effects of non-polar and polar lipid fractions

HPLC-ELSD analysis of non-polar and polar lipid fractions from WSB-extracted untreated or lipase-treated dough lipids confirmed the presence of the expected lipid classes in the fractions (Supplementary Table 1). Fig. 1 shows the specific bread volume after the non-polar and polar fractions had been added to defatted flour. Initial studies showed that the best results were obtained when 50% (60 mg) of the added lipids were unmodified flour lipids ('base lipids') and the other 50% (60 mg) were the lipid fractions under study. First of all, native 'Kolibri' flour and reconstituted flour containing unmodified wheat lipids yielded the same loaf volume confirming the suitability of the experimental approach. Further, addition of lipids enriched in non-polar lipids caused a significant bread volume decrease with a tendency that non-polar lipase-treated lipids caused an even greater decrease of the bread volume than non-polar untreated lipids. The non-polar fraction contained mainly TG, FFA, diglycerides (DG), and monoglycerides (MG). After fermentation with lipases, the FFA concentration increased strongly, whereas TG, DG and MG contents decreased. Linoleic acid is the dominant unsaturated FFA in wheat flour and forms expanded monolayers leading to instability of the liquid lamellae, and thus, low loaf volume (MacRitchie, 1976). Gaines (1966) demonstrated that double bonds in FFA prevent the close packing of monolayers, resulting in an expanded orientation. Sroan and MacRitchie (2009) concluded that differences in the gas cell stabilization characteristics of the various wheat flour lipid classes are most likely due to differences in the adsorption of monolayers at the gas–liquid interface. Linoleic acid forms expanded monolayers, while palmitic acid, a saturated FFA, forms condensed monolayers (Gaines, 1966) with no negative effect on bread loaf volume. Lipid analysis of the free and bound lipid fractions of fermented dough with lipases showed that the FFA formed were transferred to the free lipid fraction and the lyso-lipids remained in the bound lipid fraction (Gerits et al., 2014b). In this study, the free lipid fraction was obtained by hexane extraction indicating either no interaction between FFA and gluten proteins, or the interactions were restricted to the hydrophobic alkyl chain of the FFA. Consequently, the generated FFA did not contribute to aggregation of protein particles yielding stronger gluten, which is a further explanation for their deleterious functional effect in breadmaking.

In contrary, addition of lipids enriched in polar lipids caused a significant bread volume increase. These results are in accordance with the literature (Gerits et al., 2014b; Sroan and MacRitchie, 2009; MacRitchie and Gras, 1973). It is generally accepted that polar lipids contribute to the stabilization of gas cells and bread volume increase. Polar lipase-treated lipids caused a significantly higher bread volume compared to polar untreated lipids (Fig. 1). The main polar lipase reaction products are monogalctosyl monoglycerides (MGMG), N-acyl lysophosphatidyl ethanolamine (NALPE), digalactosyl monoglycerides (DGMG) and lysophosphatidyl choline (LPC), which are all capable of forming lamellar or hexagonal I mesophases (Selmair, 2010). Lipases probably catalyze the transition of lipid classes forming the detrimental hexagonal II mesophase into lipid classes promoting the lamellar and hexagonal I mesophases, which are considered to be highly effective in stabilizing gas cells (Gerits et al., 2014b).

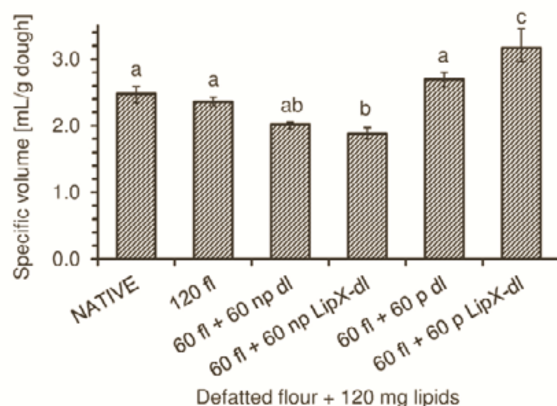

Fig. 1. Specific volume of micro-breads as affected by the addition of non-polar and polar lipid fractions. Reconstituted flour was composed of 2-propanol defatted flour (9.50 g; 8.60 g dm), 60 mg base lipids (flour lipids extracted with WSB, 20 °C) and 60 mg lipid fractions. Error bars represent mean standard deviations of triplicate determinations. Mean values associated with different letters are significantly different (one-way ANOVA, Tukey test, $p \leq 0.05$). NATIVE, untreated control flour; fl, flour lipids; dl, dough lipids; LipX-dl, Lipopan Xtra-treated (130 mg/kg flour) dough lipids; np, non-polar; p, polar.

3.3. Functional effects of specific lipid classes

The polar fractions of lipase-treated (Lipopan Xtra 130 mg/kg flour) and untreated dough lipids were further fractionated by column chromatography. Sub-fractions enriched in specific lipid classes were obtained. Six and eight polar lipid fractions were isolated from lipase-treated and untreated polar lipids, respectively. The compositions are given in Table 1. Different numbers of fractions were obtained with or without lipase because the enzyme changed the lipid composition of the polar fraction, and, for example, monogalactosyl diglycerides (MGDG) were not present any more in the lipase-treated lipid fractions. The different lipid fractions (60 mg) were mixed with base lipids (60 mg flour lipids), analyzed for their lipid composition by HPLC-ELSD (Supplementary Tables 2 and 3), and studied for their functional effects. Fig. 2 shows the specific bread volume as affected by the addition of flour lipids enriched in polar lipid classes. All fractions from polar lipase-treated dough lipids except the FFA fraction increased the bread volume compared to the control (120 mg flour lipids) (Fig. 2A). MG, ceramides (CER), and acylated sterol glucosides (ASG), minor components in this fraction, did not compensate for the negative effect of FFA. At higher concentrations, MG and ASG have a positive baking potential (Selmair and Koehler, 2009). This was confirmed by the first fraction of the separation of polar untreated dough lipids (Fig. 2B) that contained MG, CER, ASG, but no FFA. This fraction caused an increase of the bread volume, indicating the negative effects of high concentrations of FFA on bread volume.

The best functional effects were found for fractions containing MGMG, digalactosyl diglycerides (DGDG), and NALPE as the main lipid classes (Fig. 2). The results allowed the conclusion that an optimal baking lipase should preferably hydrolyze MGMG and N-acyl-phosphatidyl ethanolamine (NAPE). The improved functional effects of MGMG (bread volume increase of 36%) in comparison to MGDG (22%) (Fig. 2A, B) were already noted by Selmair and Koehler (2008). The authors reported that synthetic MGMG caused the highest volume increase at all concentrations in the baking test compared to MGDG and different commercial emulsifiers. Galactolipids with one galactose and one monoacyl moiety, like MGMG, possess far better hydrophilic lipophilic balance (HLB) in comparison with the diacyl-compound MGDG. This fact demonstrates the importance of an appropriate ratio between hydrophilic and lipophilic moieties in the molecular structure of each individual surfactant for its functional effects in bread making. It should be

Table 1
Proportion of lipid classes of fractionated lipids for reconstitution baking tests. For absolute concentrations see Supplementary Tables 2–3.

Proportion of lipid classes [%] in lipid fraction of polar lipase-treated lipids (Lipopan Xtra 130 mg/kg flour)					
FFA/MG/CER/ASG	MGMG/CBR	DGDG/NAPE/MGMG/CBR	NALPE/DGDG/CBR	DGMG/DGDG/NALPE	LPC
39/32/18/12	64/36	56/26/10/8	56/40/4	63/13/23	40

Proportion of lipid classes [%] in lipid fraction of untreated dough lipids							
MG/CER/ASG	MGDG/CBR	CBR/SG	NAPE/CBR/MGMG	NAPE/CBR/DGDG	DGDG/CBR/NAPE	NALPE/DGDG/CBR	DGDG/DGMG
16/40/44	91/9	66/34	35/37/28	58/19/23	68/13/18	54/37/10	51/49

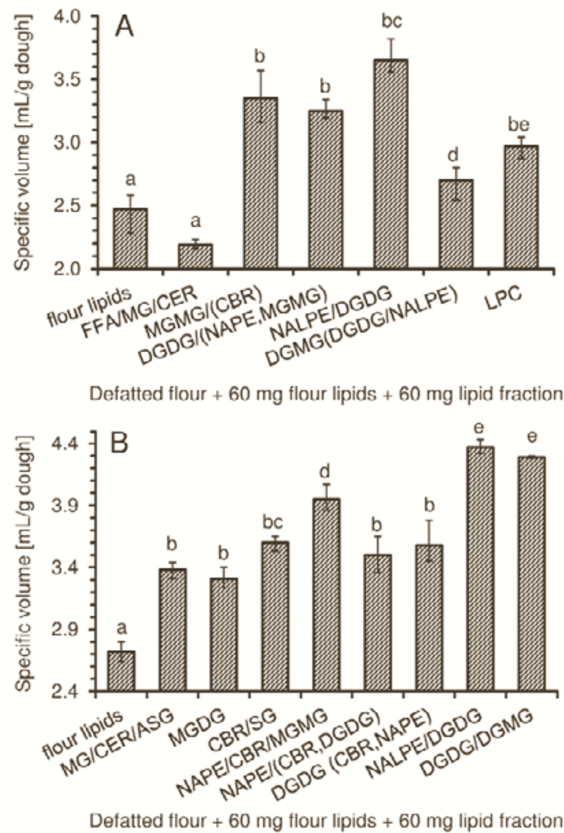

Fig. 2. Specific volume of micro-breads as affected by the addition of mixtures of polar lipid classes. Reconstituted flour was composed of 2-propanol defatted flour (9.50 g; 8.60 g dm), 60 mg base lipids (flour lipids extracted with WSB, 20 °C) and 60 mg mixtures of polar lipid classes. A: Mixtures of lipid classes of polar Lipopan Xtra-treated (130 mg/kg flour) dough lipids; B: Mixtures of lipid classes of polar untreated dough lipids. Error bars represent mean standard deviations of triplicate determinations. Mean values associated with different letters are significantly different (one-way ANOVA, Tukey test, $p \leq 0.05$). FFA, free fatty acids; MG, monoglycerides; CER, ceramides; ASG, acylated sterol glucosides; SG, sterol glucosides; CBR, cerebrosides; MGDG, monoglactosyl diglycerides; NAPE, N-acyl phosphatidyl ethanolamine; MGMG, monogalactosyl monoglycerides; DGDG, digalactosyl diglycerides; NALPE, N-acyl lysophosphatidyl ethanolamine; DGMG, digalactosyl monoglycerides; LPC, lysophosphatidyl choline. Minor components are given in brackets.

noted that Selmair and Koehler (2008) added lipids to native flour and not to defatted flour. The comparable effect found in the current work might be due to the fact that we used the MGMG-containing lipid fraction in combination with unmodified dough lipids, which were also present in the reported study (Selmair and Koehler, 2008). However, our concentrations of MGMG were much lower thus providing a lower percentage of volume increase (36% vs. 49% reported by Selmair and Koehler (2008)).

DGDG, the main galactolipid class in native flour lipids, provided a bread volume increase of 32% compared to 9% caused by its product DGMG (Fig. 2 A), showing again the requirement of a reasonable HLB, i.e. two fatty acid residues in a galactolipid with a disaccharide unit. In this term, our recent hypothesis, that the degradation of DGDG can be considered unfavorable because the functional effects of DGDG are much superior to those of DGMG (Schaffarczyk et al., 2014), has been confirmed. However, the fraction enriched with DGDG and DGMG in a molar proportion of 1:1 yielded one of the highest bread volumes (Fig. 2B). Obviously, a synergistic effect was present at comparable concentrations of both DGDG and DGMG. With increasing DGMG and decreasing DGDG concentration, the bread volume became lower (Fig. 2A). These synergistic effects between lipids are probably based on the indirect mechanism of lipids on the stabilization of the gas cell interface. One lipid-lipid interaction in dough is that deleterious non-polar lipids are emulsified as oil droplets by polar lipids such as DGMG promoting the formation of the hexagonal I mesophase (Gerits et al., 2014b; Selmair, 2010). Hence, DGMG might help in removing deleterious lipids from the interface thus supporting the effect of DGDG at the interface. Another possibility is a complementary effect of lipid classes forming different types of mesomorphic phases (Sroan and MacRitchie, 2009), like hexagonal II (MG, MGDG, NAPE) and hexagonal I (DGMG). Monolayers containing a suitable mixture of these lipid classes are of comparable stability to monolayers built of lipids favoring the lamellar type (DGDG). Finally, lipid-lipid interactions due to synergistic effects can also reduce the energy barrier of liposomes or micelles to adsorb into the interface by formation of mixed micelles with different lipid mesomorphy types. This would increase the availability of surface-active material at the interface.

The results also showed that the lipase-mediated generation of NALPE from NAPE has to be considered positive (Fig. 2 A, B) regarding the improvement of functional effects. The negative effect of NAPE in baking and the positive effect of ASG and CBR have already been described by Selmair (2010). Altogether, the influence of the major endogenous glycolipid classes and other lipid classes from lecithin added to flour on the baking performance as determined by Selmair (2010) is in agreement with our reconstitution baking tests. Despite the fact that Selmair and Koehler (2008), (2009) used a different baking test (high speed mixing after addition of lipid classes to native flour) than this study (reconstitution baking test where lipid classes were enriched, but the total lipid content remained unchanged), similar results were obtained.

Since it is difficult to suggest an optimal HLB value to predict the best functional effects of all lipid classes, the most probable mode of

action is the direct influence of the lipid classes on the gas cell interface through direct adsorption and formation of condensed monolayers. Gerits et al. (2014b) suggested that the improved gas cell stabilization caused by the action of lipases is probably based on the increase in the levels of lipids favoring the hexagonal phase I and the lamellar phase as well as on the decrease of the content of those lipid classes promoting the deleterious hexagonal phase II. Our reconstitution baking tests confirmed the theoretical mechanism of baking lipases, since the lipase reaction products (MGDG → MGMG, NAPE → NALPE) mainly contributed to the bread volume by formation of the lamellar mesophase. Also synergistic effects need to be taken into consideration, as the formation of DGMG only has an improving effect in combination with other lipids promoting the lamellar or/and hexagonal phase I. This denotes the importance of the optimum lipid composition and the breakdown of lipids to obtain an increase of the bread volume.

3.4. Synergistic effects of non-polar and polar lipids

It has been shown before that polar lipids improve the bread volume of wheat dough. To get information if a certain amount of non-polar lipids in combination with polar lipids is required to achieve an optimal improver effect, the total amount of lipid added in the reconstitution baking test was kept constant at 120 mg/10 g flour, but the proportions of non-polar and polar lipids, either from untreated or lipase-treated dough or from flour was modified. No flour lipids as base lipids were added. Fig. 3 shows the results. First of all, Fig. 3 shows that the polar and non-polar fractions of flour lipids (60 mg polar + 60 mg non-polar flour lipids) and dough lipids (60 mg polar + 60 mg non-polar dough lipids) caused similar volume increases compared to the control bread (120 mg flour lipids) as the composition of flour and dough lipids previously had been found not to be significantly different (Schaffarczyk et al., 2014). Increasing the proportion of non-polar flour or dough lipids led to a decrease of the bread volume. In contrast to the reconstitution baking test with 50% base lipids and 50% polar lipids (Fig. 1), 100% polar untreated dough lipids were more active (volume increase about 70%) compared to lipase-treated ones (35% increase). The combination of 30 mg nonpolar (lipase-treated or untreated) dough lipids and 90 mg polar lipase-treated dough lipids tended to increase the bread volume compared to the polar lipids alone. It appears that the polar lipid reaction products of baking lipases need some background lipids or non-polar lipids to provide optimal functional effects. Further experiments with different combinations of base lipids (flour lipids) and polar lipase-treated dough lipids (data not shown) emphasized that 100% polar lipase-treated lipids were less active than a combination of 50% polar lipase-treated lipids plus 50% unmodified flour lipids.

To exclude that using the fractionation-reconstitution approach had an impact on flour constituents and functionality, i.e. on gluten proteins or the native location of the endogenous lipids and, thus, on lipid–protein interactions (e.g. the phenomenon of 'lipid-binding'), we quantitated the composition of free and bound lipids of recombined flours in comparison to lipids of native and lipase-treated flours (data not shown). Free lipids of ≈9 g lyophilized recombined dough were extracted through stirring with n-hexane (2 × 25 mL, 2 × 8 h, RT). The remaining bound lipids were extracted with WSB (40 mL, 16 h, RT).) No significant differences in the behavior of lipids in recombined flour compared to native flour in regard to lipid binding was observed, confirming the suitability of our baking system based on reconstituted flour.

Further, the total amount of added lipid in the reconstitution baking test was also modified. Loaf volume trends observed on incremental addition of different lipid types (0–200 mg) showed significant differences within each lipid fraction (Fig. 4). Flour lipids (Fig. 4A) and dough lipids (Fig. 4B) gave similar results: Adding the polar lipids fraction back to defatted flour, caused a decrease of the bread volume at low lipid levels followed by an increase at higher levels. The bread volumes with high amounts of polar lipids were comparable to the result using defatted flour without lipid addition. The whole lipid fraction added back to defatted flour decreased the bread volume to a minimum at a lipid content intermediate between the defatted and untreated flour. The bread volume increased to the comparable volume of untreated flour after adding back the original amount of extracted lipids (120 mg) to defatted flour. A further increase in the level of extracted lipids brought a concurrent increase in bread volume. The non-polar lipid fraction produced a progressive decline in loaf volume. The results of the addition of flour lipids and their polar and non-polar fractions corresponded qualitatively with the classical work of MacRitchie and Gras (1973). The fact that high amounts of polar lipids yielded a bread volume, which was not higher than the bread volume of defatted flour, is probably due to the different defatting (base flour) and extraction method (lipid fractions) used. As doughs prepared from defatted flours can expand, it must be assumed that surface-active lipids are not essential for gas retention because the

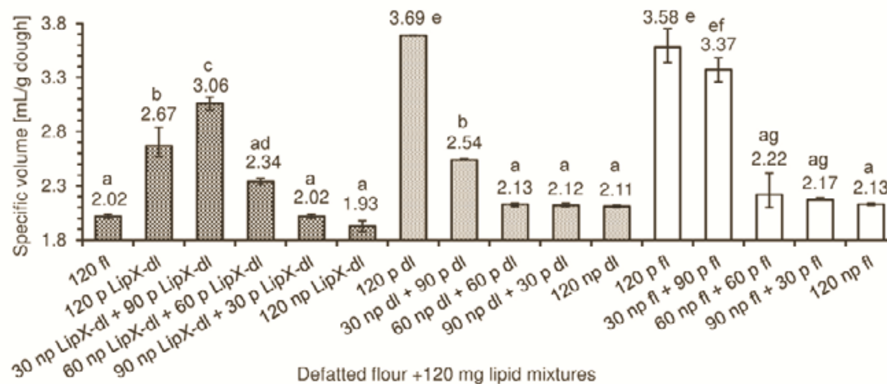

Fig. 3. Specific volume of micro-breads as affected by the proportion of non-polar and polar lipid fractions. Reconstituted flour was composed of 2-propanol defatted flour (9.50 g; 8.60 g dm) and 120 mg lipid mixtures. Error bars represent mean standard deviations of triplicate determinations. Mean values associated with different letters are significantly different (one-way ANOVA, Tukey test, $p \leq 0.05$). fl, flour lipids; dl, dough lipids; LipX-dl, Lipopan Xtra-treated (130 mg/kg flour) dough lipids; np, non-polar; p-polar.

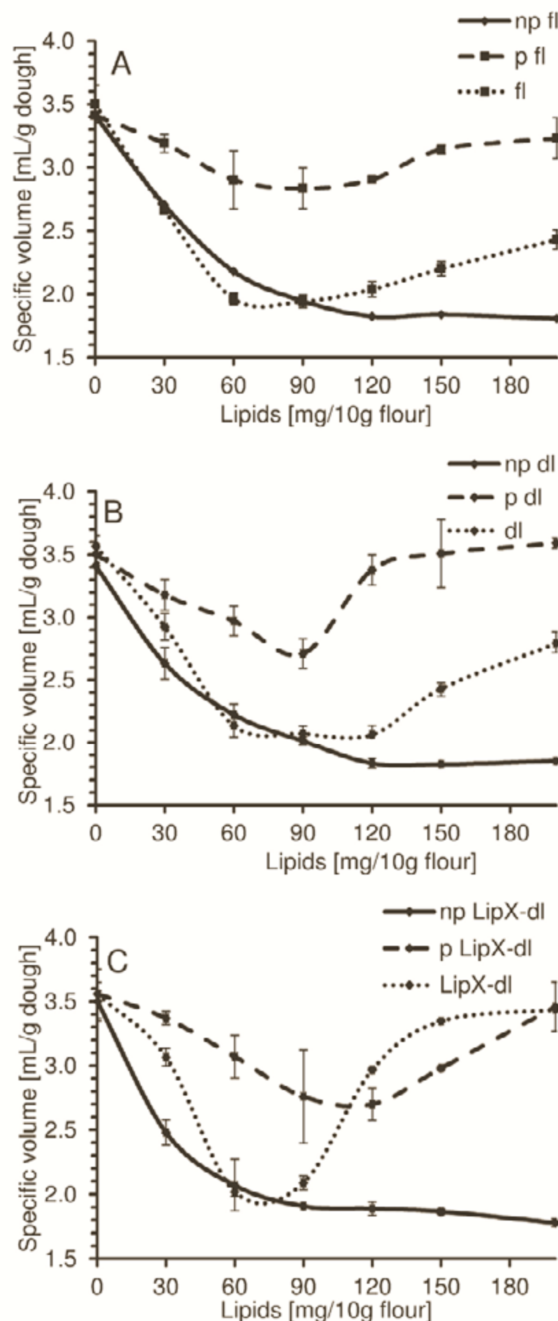

Fig. 4. Specific volume of micro-breads as affected by increasing amounts of lipids and lipid fractions. Lipids and lipid fractions (0–200 mg) were added to 2-propanol defatted flour (9.50 g; 8.60 g dm). A: np fl, non-polar flour lipids; p fl, polar flour lipids; fl, flour lipids; B: np dl, non-polar dough lipids; p dl, polar dough lipids; dl, dough lipids; C: np LipX-dl; non-polar lipase treated lipids; p LipX-dl, polar lipase-treated lipids; LipX-dl, lipase-treated lipids (130 mgLipopan Xtra/kg flour). Error bars represent mean standard deviations of duplicate determinations.

proteins dissolved in the dough aqueous phase can take over the function of lipids in defatted flour (MacRitchie and Gras, 1973; Gan et al., 1995). In defatted flour, gas cells are only stabilized by surface-active proteins resulting in a higher loaf volume and a finer and more uniform crumb structure. Proteins provide a highly condensed state of a single component and do not desorb from the gas–liquid interface easily. Addition of lipids or lipid fraction back to defatted flour causes a destabilization of protein films by formation of mixed monolayers. Salt et al. (2006) showed that pure protein interfaces are highly surface-elastic and more stable compared to mixed protein-lipid interfaces. Further addition of lipids, especially polar lipids, results in formation of lipid-dominated films.

Finally, lipase-treated dough lipids and the respective polar and non-polar fractions were also added in increasing concentrations (Fig. 4C). After adding back the original amount (120 mg/10 g flour) of the whole fraction of lipase-treated lipids a volume increase of 45% was observed compared to the whole flour/dough lipids (Fig. 4A/B). Interestingly, the incremental addition of polar lipase-treated lipids caused volume decrease to a minimum at 120 mg lipids/10 g flour, and overall they were less active than polar untreated lipids. This experiment confirmed that in contrast to polar untreated lipids, polar lipase-reaction products need non-polar background lipids for maximal volume increase. It appears that generated lipid classes promoting the hexagonal I mesophase (DGMG, LPC) give expanded monolayers leading to instability of the liquid lamellae. For optimal performance, the complementary effect of stable monolayers formed by a mixture of lipids favoring the hexagonal I and hexagonal II mesophases is needed to guarantee gas cell stability. Furthermore, the emulsification of non-polar oil droplets hinders their adsorption at the gas cell interface, thereby stabilizing the condensed monolayers of surface-active lipid classes.

3.5. Kinetic studies

Overdosing of lipase caused a decrease of the bread volume due to extensive lipid hydrolysis by lyso-phospholipase and lyso-galactolipase activity of baking lipases (Gerits et al., 2014b; Schaffarczyk et al., 2014). Long fermentation times could also lead to hydrolysis of the generated lyso-compounds leading to volume decrease. Moayedallaie et al. (2010) compared the functional and technological effects of different generations of lipases and DATEM. All lipases except Lipopan Xtra provided bread volume increase after shorter and longer fermentation times. The authors explained the bread volume decrease caused by Lipopan Xtra after long fermentation time by longer action and, thus, more extensive lipid hydrolysis as compared to the other lipases. Against this background, we quantitated the lipid composition of wheat dough after incubation with four different lipases (Lipopan Xtra, Lipopan F, Panamore Golden, and EL 2013 000 405) at optimal concentration as affected by the fermentation time (0–120 min). The results are shown in Supplementary Tables 4–7. All in all, the examined lipases yielded very similar lipid compositions. After a fermentation time of 60 min the maximum concentration of beneficial lipase reaction products were reached for all lipases. For this fermentation time, an optimum baking performance was expected from the lipid profiles showing that the concentration of the reaction products MGMG and NALPE reached their maxima. After prolonged incubation the generated lyso-galactolipids and lyso-phospholipids were further hydrolyzed upon overproofing. The generation of MGMG by lipases is limited by the natural concentration of the educts MGDG in flour. All four lipases hydrolyzed MGDG to a minimum of 0.07 mmol/kg flour corresponding to a maximum MGMG concentration obtained after 60–80 min of fermentation. With prolonged fermentation

time MGMG was further hydrolyzed due to the lyso-galactolipase activity of lipases.

Regarding partial hydrolysis of DGDG and formation of an optimal molar ratio of DGDG : DGMG of 1:1, Lipopan Xtra met the criteria. Lipopan Xtra hydrolyzed DGDG to a lower extent than the other lipases and provided an equimolar mixture of DGDG and DGMG at a fermentation time of 60 min. However, it is questionable if this synergistic effect also occurs at the naturally lower occurring concentrations of these lipid classes in wheat flour. Only Lipopan F showed lyso-galactolipase activity towards DGMG. Here, DGMG was probably a transient intermediate and was subjected to further hydrolysis by lipases. The hydrolysis of NAPE and the corresponding generation of NALPE were comparable for all lipase samples. Also, NALPE was further hydrolyzed upon overproofing. Therefore a baking lipase with high phospholipase activity may lead to complete hydrolysis of NAPE causing increased gas cell stabilization.

Altogether, the quantitation of the lipid classes showed that overproofing of lipase-containing dough also decreased the level of lipids forming a lamellar mesophase thus providing inferior stabilization of gas cells. Beside the incubation time, the lipase concentration was also of major importance for the optimal lipid composition and the breakdown of lipids to obtain bread volume increase (data no shown).

4. Conclusion

The activity-guided lipid fractionation of lipase-treated lipids showed that polar lipase-reaction products were responsible for their positive functional effects in wheat breadmaking. Reconstitution baking tests confirmed the theoretical mechanism of baking lipases, since the lipase reaction products mainly contributed to the bread volume increase by promoting formation of the lamellar mesophase. The results allowed the conclusion that an optimal baking lipase preferably hydrolyzes MGDG and NAPE, but has only moderate activity towards DGDG. Synergistic effects of the generated lipid classes with non-polar lipids might play a key role in the mechanism of baking lipases. We assume that the polar reaction products of baking lipases need the presence of unmodified base lipids or non-polar lipids to provide the optimal functional effects by the complementary effect of stable monolayers formed by a mixture of lipids favoring the hexagonal I and hexagonal II mesophases. Although these experiments have advanced the knowledge on the functional effects of lipase-treated lipids, we have to admit that in particular the reasons for the synergistic effects of polar lipase-treated lipids and non-polar lipids are still not completely understood.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcs.2016.01.007.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Lipid composition of flour lipids (fl), dough lipids (dl) and lipase-treated lipids (LipX-dl) (Lipopan Xtra 130 mg/kg flour) before and after fractionation by silica gel batch procedure into non-polar (np) and polar (p) fractions

Lipid class[a]	Concentration[b] [g/100 g] in fraction								
	fl	np fl	p fl	dl	np dl	p dl	LipX-dl	np LipX-dl	p LipX-dl
TG	33.3 ± 1.4	66.7 ± 1.4		35.5 ± 1.3	64.7 ± 0.5		28.2 ± 0.8	57.6 ± 1.9	
FFA	6.7 ± 0.6	14.0 ± 0.7		3.1 ± 0.1	6.0 ± 0.1		11.6 ± 0.2	22.6 ±0.8	
DG	6.0 ± 1.1	14.1 ± 0.6		9.9 ± 0.6	17.9 ± 0.7		3.2 ± 0.0	8.0 ± 0.8	
MG	1.5 ± 0.1	2.4 ± 0.0		1.3 ± 0.0	2.3 ± 0.0		1.6 ± 0.0	2.6± 0.1	
ASG	0.6 ± 0.0	1.0 ± 0.0		0.8 ± 0.2	0.9 ± 0.0	0.3 ± 0.2	0.5 ± 0.0		0.3 ± 0.1
SG	0.5 ± 0.0		1.3 ± 0.2	0.8 ± 0.2		1.6 ± 0.2	0.5 ± 0.0		0.8 ± 0.3
CBR	6.0 ± 0.6		10.8 ± 0.8	9.5 ± 2.5		15.3 ± 1.7	7.1 ± 0.1		11.8 ± 1.9
DGDG	11.0 ± 0.9		21.8 ± 2.1	10.4 ± 0.3		19.7 ± 1.3	6.0 ±0.1		13.4 ± 1.3
DGMG	1.5 ± 0.1		4.0 ± 0.1	1.7 ± 0.1		2.2 ± 0.1	3.6 ± 0.1		6.1 ± 0.2
MGDG	4.8 ± 0.4		7.3 ± 1.1	4.4 ± 0.0		7.8 ± 0.5	1.1 ± 0.0		1.1 ± 0.5
MGMG	3.7 ± 0.8		7.4 ± 0.4	3.6 ± 1.0		6.0 ± 1.5	6.3 ± 0.2		13.7 ± 1.6
NAPE	7.1 ± 0.1		12.4 ± 0.4	6.8 ± 0.3		13.5 ± 0.3	2.2 ± 0.0		4.0 ± 0.3
NALPE	4.7 ± 0.4		6.3 ± 0.8	4.5 ± 0.4		7.9 ± 0.2	5.7 ± 0.1		12.1 ± 1.3
PC	1.7 ± 1.1		2.4 ± 0.0	1.8 ± 0.1		2.5 ± 1.3	0.1 ± 0.0		0.1 ± 0.1
LPC	3.6 ± 0.5		4.6 ± 0.4	2.1 ± 0.3		5.3 ± 0.8	3.8 ± 0.1		7.8 ± 0.7

[a] TG, triglycerides; FFA, free fatty acids; DG, diglycerides; MG, monoglycerides; ASG, acylated sterol glucosides; SG, sterol glucosides; CBR, cerebrosides; DGDG, digalactosyl diglycerides; DGMG, digalactosyl monoglycerides; MGDG, monogalactosyl diglycerides; MGMG, monogalactosyl monoglycerides; NAPE, N-acyl-phosphatidyl ethanolamine; NALPE, N-acyl-lysophosphatidyl ethanolamine; PC, phosphatidyl choline; LPC, lysophosphatidyl choline

[b] mean values ± standard deviations of triplicate determinations

Supplementary Table 2. Lipid composition of mixtures consisting of 60 mg wheat flour lipids and 60 mg lipid fraction of polar lipase-treated lipids (Lipopan Xtra 130 mg/kg flour) added in reconstitution micro-baking tests. Main lipid classes in the mixtures are given in bold

Lipid class[a]	Concentration[b] [mg/120 mg lipids] in						
	Flour lipids	Lipid mixture (60 mg flour lipids + 60 mg lipid fractions)					
		FFA/MAG/ CER	MGMG/ (CBR)	DGDG/ (NAPE,MGMG)	NALPE/ DGDG	DGMG/ (DGDG/NALPE)	LPC
TG	28.1 ± 1.94	14.1 ± 1.0	14.1 ± 1.0	14.1 ± 1.0	14.1 ± 1.0	14.1 ± 1.0	14.1 ± 1.0
FFA	6.7 ± 0.2	**22.0 ± 4.8**	3.3 ± 0.1	3.3 ± 0.1	3.3 ± 0.1	3.3 ± 0.1	3.3 ± 0.1
DG	5.4 ± 1.8	**6.5 ± 2.9**	2.7 ± 0.9	2.7 ± 0.9	2.7 ± 0.9	2.7 ± 0.9	2.7 ± 0.9
MG	1.6 ± 0.1	**19.7 ± 4.3**	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
CER	2.4 ± 0.3	**11.8 ± 2.4**	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
ASG	0.4 ± 0.1	**7.1 ± 1.6**	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
SG	1.4 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
CBR	1.2 ± 0.1	0.6 ± 0.0	**22.0 ± 4.1**	5.1± 1.6	3.0 ± 0.9	2.0 ± 0.2	0.6 ± 0.0
DGDG	17.0 ± 0.4	8.5 ± 0.2	8.5 ± 0.2	**41.4 ± 7.2**	**32.8 ± 2.7**	**16.3 ± 3.1**	8.5 ± 0.2
DGMG	1.7 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	**39.3 ± 4.8**	0.8 ± 0.1
MGDG	3.7 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1
MGMG	3.0 ± 0.4	1.5 ± 0.2	**38.9 ± 4.9**	**7.6 ± 1.9**	1.5 ± 0.2	1.5 ± 0.2	1.5 ± 0.2
NAPE	7.2 ± 0.0	3.6 ± 0.0	3.6 ± 0.0	**18.7 ± 3.1**	3.6 ± 5.0	3.6 ± 0.0	3.6 ± 0.0
NALPE	4.2 ± 0.8	2.1 ± 0.4	2.1 ± 0.4	2.1 ± 0.4	**36.1 ± 6.1**	**15.3 ± 4.3**	2.1 ± 0.4
PC	1.0 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
LPC	6.7 ± 0.4	3.3 ± 0.2	3.3 ± 0.2	3.3 ± 0.2	3.3 ± 0.2	3.3 ± 0.2	**20.4 ± 2.8**

[a] TG, triglycerides; FFA, free fatty acids; DG, diglycerides; MG, monoglycerides; CER, ceramides; ASG, acylated sterol glucosides; SG, sterol glucosides; CBR, cerebrosides; DGDG, digalactosyl diglycerides; DGMG, digalactosyl monoglycerides; MGDG, monogalactosyl diglycerides; MGMG, monogalactosyl monoglycerides; NAPE, N-acyl-phosphatidyl ethanolamine; NALPE, N-acyl-lysophosphatidyl ethanolamine; PC, phosphatidyl choline; LPC, lysophosphatidyl choline

[b] mean values ± standard deviations of triplicate determinations

Supplementary Table 3. Lipid composition of mixtures consisting of 60 mg wheat flour lipids and 60 mg lipid fraction of untreated dough lipids added in reconstitution micro-baking tests. Main lipid classes in the mixtures are given in bold

Lipid class[a]	Concentration[b] [mg/120 mg lipids] in							
	Lipid mixture (60 mg flour lipids + 60 mg lipid fractions)							
	MAG/CER/ ASG	MGDG	CBR/SG	NAPE/CBR/ MGMG	NAPE (CBR,DGDG)	DGDG (CBR,NAPE)	NALPE/ DGDG	DGDG/ DGMG
TG	16.7 ± 0.7	16.7 ± 0.7	16.7 ± 0.7	16.7 ± 0.7	16.7 ± 0.7	16.7 ± 0.7	16.7 ± 0.7	16.7 ± 0.7
FFA	3.8 ± 0.1	3.8 ± 0.1	3.8 ± 0.1	3.8 ± 0.1	3.8 ± 0.1	3.8 ± 0.1	3.8 ± 0.1	3.8 ± 0.1
DG	3.7 ± 0.1	3.7 ± 0.1	3.7 ± 0.1	3.7 ± 0.1	3.7 ± 0.1	3.7 ± 0.1	3.7 ± 0.1	3.7 ± 0.1
MG	**9.9 ± 1.8**	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
CER	**24.6 ± 3.6**	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
ASG	**26.4 ± 12.2**	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
SG	0.5 ± 0.1	0.5 ± 0.1	**19.3 ± 3.4**	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
CBR	2.3 ± 0.4	7.5 ± 0.8	**39.3 ± 4.1**	**28.4 ± 3.7**	**14.3± 2.1**	**9.7 ± 2.9**	7.9 ± 0.7	2.3 ± 0.4
DGDG	6.8 ± 0.2	6.8 ± 0.2	6.8 ± 0.2	6.8 ± 0.2	6.8 ± 0.2	**47.2 ± 9.8**	**28.4 ± 0.8**	**34.4 ±9.9**
DGMG	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	**28.4 ± 4.0**
MGDG	2.3 ± 0.4	**54.6 ± 9.9**	2.3 ± 0.4	2.3 ± 0.4	2.3 ± 0.4	2.3 ± 0.4	2.3 ± 0.4	2.3 ± 0.4
MGMG	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	**21.9 ± 4.2**	**16.0 ± 4.4**	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.1
NAPE	3.6 ± 0.1	3.6 ± 0.1	3.6 ± 0.1	**28.2 ± 4.4**	**39.5 ± 0.2**	**14.4 ± 2.8**	3.6 ± 0.1	3.6 ± 0.1
NALPE	2.2 ± 0.1	2.2 ± 0.1	2.2 ± 0.1	2.2 ± 0.1	2.2 ± 0.1	2.2 ± 0.1	**34.0 ± 1,2**	2.2 ± 0.1
PC	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
LPC	2.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.1

[a] TG, triglycerides; FFA, free fatty acids; DG, diglycerides; MG, monoglycerides; CER, ceramides; ASG, acylated sterol glucosides; SG, sterol glucosides; CBR, cerebrosides; DGDG, digalactosyl diglycerides; DGMG, digalactosyl monoglycerides; MGDG, monogalactosyl diglycerides; MGMG, monogalactosyl monoglycerides; NAPE, N-acyl-phosphatidyl ethanolamine; NALPE, N-acyl-lysophosphatidyl ethanolamine; PC, phosphatidyl choline; LPC, lysophosphatidyl choline

[b] mean values ± standard deviations of triplicate determinations

Supplementary Table 4. Concentration of lipid classes in lipids from wheat dough after addition of the lipase Lipopan Xtra (130 mg/kg flour) as affected by the fermentation time

Lipid class[a]	Control[b]	Concentration[c] [mg/kg flour dm] at fermentation time [min]									
		0	10	20	30	40	50	60	80	100	120
TG	3733 ± 45	3700 ± 86	3565 ± 86	3399 ± 93	3079 ± 16	2961 ± 95	2986 ± 63	2902 ± 50	2875 ± 95	3018 ± 62	2841 ± 23
FFA	376 ± 6	513 ± 16	592 ± 62	729 ± 34	756 ± 68	742 ± 65	741 ± 42	862 ± 11	851 ± 37	1015 ± 91	929 ± 41
DG	1815 ± 9	1059 ± 28	910 ± 78	806 ± 49	916 ± 59	583 ± 11	517 ± 16	547 ± 13	341 ± 20	436 ± 99	730 ± 79
MG	109 ± 8	151 ± 42	104 ± 10	118 ± 5	112 ± 3	128 ± 33	135 ± 41	128 ± 7	104 ± 6	154 ± 22	134 ± 2
SG	144 ± 1	156 ± 18	108 ± 23	148 ± 20	117 ± 13	123 ± 30	113 ± 10	104 ± 17	89 ± 13	132 ± 19	155 ± 57
CBR	1485 ± 32	1533 ± 41	1138 ± 87	1780 ± 47	1930 ± 89	2000 ± 74	1421 ± 62	1435 ± 26	1315 ± 23	1667 ± 7	1920 ± 86
DGDG	1312 ± 13	1380 ± 21	1258 ± 93	1243 ± 51	1102 ± 46	1108 ± 62	933 ± 97	877 ± 52	670 ± 47	737 ± 51	825 ± 79
DGMG	119 ± 12	212 ± 31	223 ± 26	321 ± 37	505 ± 37	524 ± 92	560 ± 4	662 ± 3	607 ± 18	500 ± 43	386 ± 4
MGDG	480 ± 65	202 ± 9	114 ± 29	134 ± 20	98 ± 22	111 ± 17	112 ± 60	83 ± 29	60 ± 19	69 ± 19	77 ± 12
MGMG	200 ± 26	350 ± 46	465 ± 43	542 ± 44	603 ± 43	640 ± 27	660 ± 41	674 ± 47	707 ± 19	663 ± 39	636 ± 39
NAPE	634 ± 26	641 ± 18	577 ± 80	405 ± 47	339 ± 91	257 ± 24	259 ± 59	240 ± 21	259 ± 18	286 ± 33	324 ± 36
NALPE	446 ± 27	624 ± 70	718 ± 71	780 ± 60	791 ± 71	772 ± 57	744 ± 52	745 ± 51	739 ± 21	774 ± 66	735 ± 67
PC	102 ± 1	76 ± 25	51 ± 9	43 ± 10	42 ± 6	40 ± 21	24 ± 2	19 ± 5	12 ± 10	19 ± 3	14 ± 3
LPC	291 ± 1	354 ± 11	374 ± 41	445 ± 26	465 ± 38	453 ± 36	448 ± 27	464 ± 33	409 ± 19	419 ± 16	728 ± 33

[a] TG, triglycerides; FFA, free fatty acids; DG, diglycerides; MG, monoglycerides; ASG, acylated sterol glucosides; SG, sterol glucosides; CBR, cerebrosides; DGDG, digalactosyl diglycerides; DGMG, digalactosyl monoglycerides; MGDG, monogalactosyl diglycerides; MGMG, monogalactosyl monoglycerides; NAPE, N-acyl-phosphatidyl ethanolamine; NALPE, N-acyl-lysophosphatidyl ethanolamine; PC, phosphatidyl choline; LPC, lysophosphatidyl choline

[b] No enzyme addition, fermentation time 60 min

[c] Mean values ± standard deviations of triplicate determinations.

Supplementary Table 5. Concentration of lipid classes in lipids from wheat dough as affected by the addition of the lipase Lipopan F (70 mg/kg flour) as affected by the fermentation time

Lipid class[a]	Control[b]	Concentration[c] [mg/kg flour dm] at fermentation time [min]									
		0	10	20	30	40	50	60	80	100	120
TG	3733 ± 45	3754 ± 40	3377 ± 88	3348 ± 5	3195 ± 74	3224 ± 26	2966 ± 76	2985 ± 55	3007 ± 52	2778 ± 36	2679 ± 98
FFA	376 ± 6	513 ± 16	640 ± 56	785 ± 30	908 ± 86	851 ± 69	1012 ± 70	966 ± 97	905 ± 93	948 ± 77	995 ± 29
DG	1815 ± 9	1059 ± 28	513 ± 79	544 ± 17	807 ± 58	920 ± 12	723 ± 68	266 ± 55	358 ± 16	269 ± 64	602 ± 39
MG	109 ± 8	151 ± 42	134 ± 19	167 ± 7	137 ± 9	119 ± 35	148 ± 30	208 ± 44	119 ± 19	263 ± 67	226 ± 14
SG	144 ± 1	156 ± 18	104 ± 8	167 ± 14	145 ± 2	145 ± 38	110 ± 14	101 ± 20	93 ± 18	137 ± 19	128 ± 23
CBR	1485 ± 32	1533 ± 41	1062 ± 85	1848 ± 64	1850 ± 50	1812 ± 87	1737 ± 58	1464 ± 71	927 ± 60	1492 ± 87	1801 ± 69
DGDG	1312 ± 13	1380 ± 21	983 ± 75	968 ± 1	879 ± 30	802 ± 46	755 ± 86	436 ± 55	342 ± 86	342 ± 56	331 ± 27
DGMG	119 ± 12	212 ± 31	305 ± 6	559 ± 33	555 ± 21	613 ± 17	541 ± 35	556 ± 40	541 ± 30	500 ± 24	524 ± 24
MGDG	480 ± 65	254 ± 68	131 ± 16	136 ± 30	119 ± 18	125 ± 48	77 ± 19	91 ± 11	56 ± 9	52 ± 7	62 ± 15
MGMG	200 ± 26	406 ± 38	524 ± 35	597 ± 18	658 ± 2	630 ± 45	677 ± 48	671 ± 39	599 ± 41	587 ± 40	515 ± 8
NAPE	634 ± 26	641 ± 18	549 ± 14	551 ± 85	570 ± 28	449 ± 89	327 ± 73	280 ± 58	252 ± 60	290 ± 54	319 ± 13
NALPE	446 ± 27	594 ± 74	628 ± 41	738 ± 39	810 ± 37	835 ± 54	814 ± 58	762 ± 66	718 ± 99	649 ± 76	667 ± 28
PC	102 ± 1	76 ± 25	36 ± 7	28 ± 1	25 ± 15	19 ± 2	20 ± 13	19 ± 12	18 ± 10	13 ± 1	17 ± 11
LPC	291 ± 1	354 ± 11	406 ± 6	435 ± 28	487 ± 2	467 ± 17	499 ± 7	508 ± 15	444 ± 25	432 ± 17	356 ± 14

[a] TG, triglycerides; FFA, free fatty acids; DG, diglycerides; MG, monoglycerides; ASG, acylated sterol glucosides; SG, sterol glucosides; CBR, cerebrosides; DGDG, digalactosyl diglycerides; DGMG, digalactosyl monoglycerides; MGDG, monogalactosyl diglycerides; MGMG, monogalactosyl monoglycerides; NAPE, N-acyl-phosphatidyl ethanolamine; NALPE, N-acyl-lysophosphatidyl ethanolamine; PC, phosphatidyl choline; LPC, lysophosphatidyl choline
[b] No enzyme addition, fermentation time 60 min
[c] Mean values ± standard deviations of triplicate determinations.

Supplementary Table 6. Concentration of lipid classes in lipids from wheat dough as affected by the addition of the lipase Panamore Golden (70 mg/kg flour) as affected by the fermentation time

Lipid class[a]	Control[b]	Concentration[c] [mg/kg flour dm] at fermentation time [min]									
		0	10	20	30	40	50	60	80	100	120
TG	4325 ± 53	4169 ± 58	4102 ± 67	3806 ± 28	3776 ± 35	3720 ± 42	3210 ± 19	3028 ± 53	3137 ± 93	3110 ± 68	3169 ± 90
FFA	388 ± 39	613 ± 61	743 ± 27	897 ± 51	955 ± 34	1005 ± 79	1012 ± 47	1024 ± 20	1128 ± 69	1112 ± 58	1236 ± 66
DG	464 ± 68	416 ± 52	688 ± 15	583 ± 18	659 ± 50	526 ± 29	651 ± 67	776 ± 53	840 ± 39	888 ± 80	814 ± 53
MG	166 ± 3	112 ± 2	143 ± 2	171 ± 5	146 ± 12	158 ± 15	148 ± 4	148 ± 19	159 ± 21	174 ± 12	195 ± 5
SG	53 ± 15	79 ± 11	92 ± 16	82 ± 10	58 ± 13	77 ± 13	71 ± 4	53 ± 7	76 ± 4	62 ± 12	79 ± 15
CBR	1121 ± 58	1305 ± 36	1520 ± 74	2177 ± 47	2040 ± 37	1948 ± 77	2017 ± 36	2112 ± 76	2396 ± 62	1522 ± 58	2752 ± 28
DGDG	1248 ± 27	1103 ± 85	1039 ± 53	888 ± 27	550 ± 67	470 ± 54	443 ± 46	317 ±20	258 ± 16	252 ± 21	241 ± 25
DGMG	137 ± 11	255 ± 46	441 ± 26	660 ± 24	740 ± 43	796 ± 29	799 ± 47	903 ± 14	909 ± 41	931 ± 21	997 ± 30
MGDG	453 ± 20	213 ± 68	134 ± 9	91 ± 42	61 ± 22	67 ± 25	58 ± 9	67 ± 28	44 ± 5	70 ± 42	76 ± 9
MGMG	183 ± 5	380 ± 23	508 ± 31	560 ± 33	631 ± 30	612 ± 41	654 ± 50	608 ± 23	554 ± 46	481 ± 42	475 ± 40
NAPE	786 ± 19	634 ± 47	613 ± 59	474 ± 71	265 ± 3	206 ± 53	256 ± 24	237 ± 16	256 ± 1	249 ± 18	282 ± 10
NALPE	420 ± 21	508 ± 15	741 ± 42	781 ± 46	753 ± 54	770 ± 35	809 ± 66	812 ± 5	846 ± 67	789 ± 72	661 ± 27
PC	158 ± 16	83 ± 3	92 ± 6	98 ± 5	73 ± 2	87 ± 10	78 ± 6	72 ± 1	76 ± 4	81 ± 6	82 ± 9
LPC	395 ± 23	423 ± 18	435 ± 12	428 ± 32	433 ± 17	435 ± 32	441 ± 18	442 ± 12	448 ± 28	457 ± 33	449 ± 39

[a] TG, triglycerides; FFA, free fatty acids; DG, diglycerides; MG, monoglycerides; ASG, acylated sterol glucosides; SG, sterol glucosides; CBR, cerebrosides; DGDG, digalactosyl diglycerides; DGMG, digalactosyl monoglycerides; MGDG, monogalactosyl diglycerides; MGMG, monogalactosyl monoglycerides; NAPE, N-acyl-phosphatidyl ethanolamine; NALPE, N-acyl-lysophosphatidyl ethanolamine; PC, phosphatidyl choline; LPC, lysophosphatidyl choline
[b] No enzyme addition, fermentation time 60 min
[c] Mean values ± standard deviations of triplicate determinations.

Supplementary Table 7. Concentration of lipid classes in lipids from wheat dough as affected by the addition of the lipase EL 2013 000 405 (70 mg/kg flour) as affected by the fermentation time

Lipid class[a]	Control[b]	Concentration[c] [mg/kg flour dm] at fermentation time [min]									
		0	10	20	30	40	50	60	80	100	120
TG	4325 ± 53	4047 ± 21	3867 ± 19	3722 ± 86	3748 ± 61	3331 ± 53	3114 ± 56	3254 ± 59	3137 ± 75	3236 ± 91	3062 ± 76
FFA	388 ± 39	704 ± 8	750 ± 70	824 ± 31	879 ± 24	976 ± 90	1006 ± 91	1101 ± 75	1145 ± 90	1193 ± 69	1178 ± 57
DG	464 ± 68	412 ± 76	519 ± 54	426 ± 42	535 ± 66	655 ± 57	654 ± 67	568 ± 25	815 ± 75	865 ± 76	791 ± 43
MG	166 ± 3	134 ± 12	145 ± 9	153 ± 8	152 ± 11	158 ± 14	163 ± 13	155 ± 13	171 ± 14	181 ± 18	179 ± 3
SG	53 ± 15	86 ± 13	68 ± 12	46 ± 30	54 ± 20	70 ± 9	63 ± 5	58 ± 14	69 ± 14	70 ± 12	53 ± 19
CBR	1121 ± 58	1509 ± 36	1509 ± 68	1538 ± 57	1850 ± 24	1996 ± 51	2110 ± 73	2041 ± 70	2883 ± 62	1688 ± 69	1404 ± 79
DGDG	1248 ± 27	1031 ± 64	845 ±66	529 ± 81	470 ± 71	460 ± 66	356 ± 59	265 ± 38	213 ± 16	226 ± 30	190 ± 9
DGMG	137 ± 11	327 ± 17	503 ± 37	650 ± 35	745 ± 35	743 ± 36	778 ± 8	895 ± 56	942 ± 41	981 ± 49	986 ± 38
MGDG	453 ± 20	162 ± 4	58 ± 10	49 ± 14	53 ± 24	51 ± 20	50 ± 9	46 ± 3	46 ± 5	50 ± 8	44 ± 1
MGMG	181 ± 5	398 ± 25	490 ± 13	548 ± 29	605 ±41	634 ± 34	645 ± 46	678 ± 22	592 ± 49	533 ± 50	448 ± 37
NAPE	786 ± 19	571 ± 78	457 ± 48	315 ± 38	254 ± 22	283 ± 47	263 ± 14	275 ± 23	258 ± 16	260 ± 60	302 ± 70
NALPE	420 ± 21	574 ± 8	552 ± 32	603 ± 32	686 ± 46	717 ± 50	869 ± 67	856 ± 60	803 ± 73	698 ± 63	606 ± 45
PC	158 ± 16	92 ± 5	92 ± 5	78 ± 2	81 ± 3	73 ± 5	70 ± 2	78 ± 7	78 ± 7	82 ± 10	79 ± 3
LPC	395 ± 23	420 ± 14	435 ± 31	428 ± 9	427 ± 16	425 ± 24	433 ± 25	447 ± 37	451 ± 23	447 ± 45	449 ± 25

[a] TG, triglycerides; FFA, free fatty acids; DG, diglycerides; MG, monoglycerides; ASG, acylated sterol glucosides; SG, sterol glucosides; CBR, cerebrosides; DGDG, digalactosyl diglycerides; DGMG, digalactosyl monoglycerides; MGDG, monogalactosyl diglycerides; MGMG, monogalactosyl monoglycerides; NAPE, N-acyl-phosphatidyl ethanolamine; NALPE, N-acyl-lysophosphatidyl ethanolamine; PC, phosphatidyl choline; LPC, lysophosphatidyl choline

[b] No enzyme addition, fermentation time 60 min

[c] Mean values ± standard deviations of triplicate determinations.

4. General discussion

The study was divided into three parts that were implemented one by one: 1) the quantitation of the altered lipid composition of lipase-treated wheat lipids by HPLC-ELSD; 2) establishment of a micro-reconstitution baking test that provides the same results as native wheat flour in terms of functional effects and allows studying the functional effects of any type of lipid in breadmaking; 3) determination of the relationships between specific wheat lipid classes and their functional effects in wheat bread.

Composition of lipids in wheat dough as affected by lipase modification

The quantitation of lipase reaction products with an optimized HPLC-ELSD procedure was performed in the first part of the study. The ternary gradient was optimized in numerous steps to be able to separate 27 lipid classes (Schaffarczyk et al., 2014). Drawbacks of such a procedure are the irregular baseline due to several solvent mixtures, especially with increasing water concentration during the gradient and that two different sensitives of the ELSD have to be used due to the wide range of concentrations of different lipid classes naturally occurring in wheat lipids. The latter can be easily solved by using an ELSD-detector or suitable software which allows altering the signal gain during the run, hence, avoiding multiple injections. Quantitative methods require reference compounds for calibration and all relevant lipid classes from wheat have to be present. For this purpose, pure lipid classes were isolated from wheat flour lipids to obtain reference compounds for HPLC-ELSD analysis. In particular, DGMG and NALPE were not commercially available and had to be laboriously isolated by a batch procedure and column chromatography. It was demonstrated that lipases can also be used in a buffered system to generate lipase reaction products. Here, this buffered system was used to generate sufficient amounts of DGMG from isolated DGDG. In the end, all required reference compounds for the quantitation of lipid classes were present in amounts of 9 - 650 mg (Schaffarczyk et al., 2014). Firstly, a suitable solvent for the quantitative extraction of lipids from wheat flour and dough had to be found. WSB and 2-propanol (75 °C) were used to extract wheat flour and lyophilized dough powder. The extracts were analyzed by the novel HPLC-method with ELSD detection. The evaluation of the quantitative data showed that more glycolipids and phospholipids were extracted using WSB compared to 2-propanol (75 °C) due to the ‘bi-polar’ character of WSB. Moreover, the lipid composition

after incubation with different concentrations of four baking lipases and increasing fermentation times (0-120 min) were quantitated. With increasing amount of lipases, a decrease of TG, DGDG, NAPE and MGDG occurred, in particular after proofing. This went hand in hand with increased concentrations of the hydrolysis products FFA, MGMG, NALPE and DGMG. However, the changes of the DGDG and DGMG concentrations revealed that the formation of DGMG did not compensate for the decrease of the DGDG concentration, especially for the lipase Lipopan F (Schaffarczyk et al., 2014). An explanation could be that DGMG is a transient intermediate and subjected to further hydrolysis by the lipase. After prolonged incubation time or increased lipase concentration the generated lyso-galactolipids and lyso-phospholipids were further hydrolyzed. Gerits et al. (2014) already showed that an overdose of lipases Lipopan F and Lecitase Ultra caused bread volume decrease caused by lyso-phospholipase and lyso-galactolipase activity depending on the substrates available.

The positive functional effects of reaction products of lipases were demonstrated with micro-baking tests based on 10 g flour. The dough was mixed in a Microfarinograph (63/min) until the optimum consistency of the dough was reached. The bread volume increase was found to correlate to enzyme dosage until a plateau was reached. Lipopan F was found to reach a plateau at a lower dosage (70 mg granulate/kg flour) compared to Lipopan Xtra (130 mg granulate/kg flour) (Schaffarczyk et al., 2014). Further increase in enzyme dosage did not cause statistically significant differences in the obtained bread volumes. The functional effects of lipases (volume increase of 56-58%) were comparable with DATEM (58%), superior to SSL (47%) and even more superior to MG (11%). Furthermore, the amount of commercial lipase product required for this effect was approximately 1/50 of the amount of surfactants added. The typical plateau phase was seen at DATEM concentrations between 0.4% and 0.8%, comparable with the plateau phase after lipase application. SSL showed the same phenomenon and caused a maximum bread volume increase of 47% at a concentration of 0.4%.

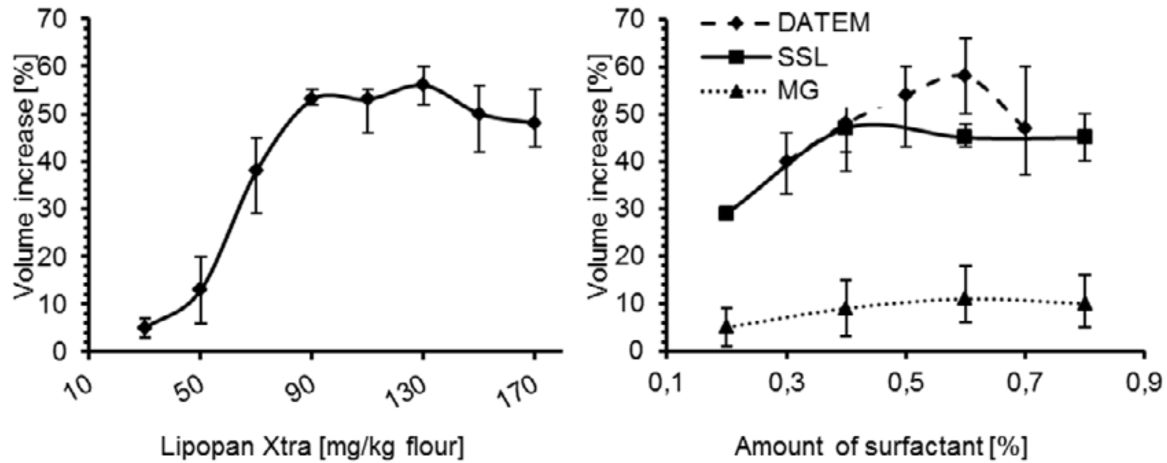

Figure 4.1: Micro-baking tests with 10 g of flour. Change of the bread volume as affected by increasing concentrations of the lipase Lipopan Xtra or the emulsifiers DATEM, SSL, and MG. Error bars represent mean standard deviations of triplicate determinations.

Previous studies on the effect of DATEM have shown that the volume increases were higher in the micro-baking test (40-60% volume increase) as compared to a normal-scale test with 300 g of flour (10-20% volume increase) (Koehler, 2001). However, the correlation between micro- and normal-scale baking test was high because the same trend of the loaf volumes was obtained with both setups. Only the absolute volume increases are higher using the micro-baking tests than the normal-scale baking tests due to the higher surface-per-weight ratio for 10 g breads than for 300 g / 1000 g breads. These results strongly suggested the micro-baking test with a Microfarinograph as the preferred test to assess the effects of lipase addition bread volume. No significant bread volume increase was detected after lipase application using a micro-rapid-mix test (MRMT), where the dough was mixed for 1 min with a high speed mixer (1250/min). It can only be speculated why the MRMT was not suitable to study the functional effect of lipases. One explanation would be that the enzymes were shear sensitive and were inactivated by the high mechanical input during mixing. In contrast, the MRMT is suitable for analysis of bread volume increase after application of DATEM. This known superior stability of DATEM in different applications compared to lipases was also demonstrated by a baking test in which the dough was mechanically stressed after the end of proofing before baking. After proofing, the dough was dropped from a height of 10 cm to stress the dough mechanically and was baked immediately. Figure 4.2 shows that breads with additives were more affected by mechanical stress than bread without any additive.

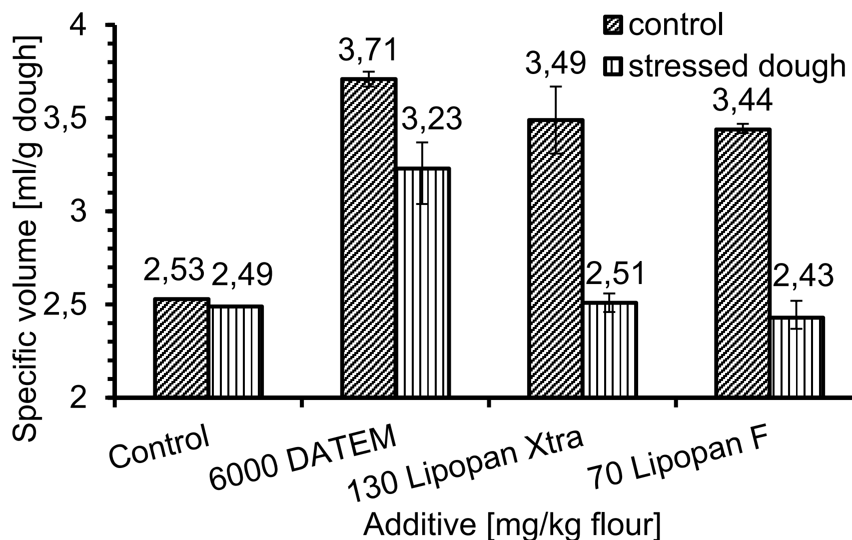

Figure 4.2: Mechanical stress-baking tests using 10 g flour and different additives. Change of the specific bread volume as affected by dropping the dough from a height of 10 cm prior to baking. Error bars represent the mean standard deviations of triplicate determinations.

Breads with DATEM or lipases have an increased gas retention capacity and are able to retain more gas. The more gas is retained, the more gas can be released caused by mechanical stress. DATEM stabilized the dough matrix better and these doughs were less affected by mechanical stress compared to lipase-containing doughs. This demonstrated the need of an improved understanding of the mechanism of action of baking lipases for developing an improved baking lipase.

Micro-scale reconstitution baking tests with defatted flour

Hence, the next part of this thesis was focused on the development of a method for fractionation and reconstitution of wheat flour to investigate the functional effects of lipase-treated wheat lipids by means of micro-scale methods and, thereby, on the second aim of the study. We had shown that WSB at 20 °C is the preferred solvent for complete lipid isolation from wheat flour and wheat dough. As we wanted to examine the functional effects of lipase-treated lipids, which differed mainly in the polar lipid portion, extraction of the lipid fraction with WSB at 20 °C was chosen. Two approaches for preparation and isolation of lipase reaction products were compared: isolation of lipase-treated dough lipids after fermentation of dough with lipases using WSB as extraction solvent and generation of lipase reaction products of WSB-extracted flour lipids in a simulated dough-system. The former procedure was successfully applied for quantitation of lipase-reaction products extracted with WSB in the first part of the study. However, preparation of the dough, freeze-drying and extraction of

the lyophilized dough was very time consuming. Therefore, WSB-isolated, unmodified flour lipids were coated on wheat starch, cellulose, SiO_2, glass beads or Al_2O_3. Phosphate-buffer (pH 5.54, 0.1 mol/L) was added to build a lipid-water-interphase. After incubation with lipases the lipase reaction products were extracted with methanol and dichloromethane and analyzed by HPLC. The results demonstrated that cellulose, SiO_2 and Al_2O_3 provided the lowest amount of lipase reaction products. Better results were obtained for wheat starch and the most similar lipid composition compared to a dough system was provided by lipid-coated glass beads. However, the lipid composition obtained after incubation wheat dough with lipase could not be reproduced with these systems. Due to the fact, that the lipid composition after incubation with lipases is strongly dependent on the system used, it made sense that lipase reaction products extracted from a 'real' dough was chosen for further investigations to exclude inconsistencies.

The most suitable method for preparing the defatted flour for reconstitution remained to be developed. Four approaches were compared: flour defatted with WSB at 20 °C, with 2-propanol at 75 °C, and with 2-propanol at 20 °C. Finally, an approach using 'synthetic' flour composed of wheat starch, wheat gluten and gelatin was used. In summary, only the reconstitution MBT using defatted flour obtained by extraction with 2-propanol at 20 °C was suitable to determine the functionality of wheat lipids in breadmaking (Schaffarczyk et al., 2016a). When lipids modified by different concentrations of the two lipases were added to the defatted flour, dose-dependent increases in loaf volume of up to 46% (Lipopan Xtra; 130 mg/kg flour) and 47% (Lipopan F; 70 mg/kg flour) were obtained. These bread volume increases were comparable to the experiments with native flour (volume increases of 47% or 56%, respectively). The other three approaches using flour defatted with WSB at 20 °C, with 2-propanol at 75 °C or 'synthetic' flour showed no significant change in bread volume as affected by the different lipid mixtures and, therefore, were not suitable to demonstrate the improving effect of lipase-treated lipids in wheat breadmaking. Proper selection of solvent and extraction temperature was of major importance to sustain the functionality of defatted flour used for recombination. Dough and gluten from flour defatted with WSB (extracted at 20 °C) and 2-propanol (extracted at 75 °C) had inferior extensibility and loaf volume compared to control flour extracted with 2-propanol at 20 °C. MixoLab data confirmed that flour extracted with 2-propanol at 20 °C was considered the most suitable defatted flour for reconstitution experiments compared to the two other extraction methods tested (Schaffarczyk et al., 2016a). Visual analysis using CLSM demonstrated that dough made from flour defatted

with 2-propanol (75°C) and WSB (20 °C) showed no protein network formation. Precipitated protein particles were detected, which were scattered and clustered but not interconnected (Schaffarczyk et al., 2016a). The fact that the protein particles were not interconnected explains why no wet gluten for extension tests could be isolated by washing the dough. It seems that the gluten particles could not be stretched and elongated by the mechanical energy during kneading. It appeared that the defatting methods had a disaggregating effect on gluten proteins either caused by a combination of solvent and heat (2-propanol, 75 °C) or by the solvent alone (WSB, 20 °C). A possible reason might be thiol-disulfide interchange reactions between gluten protein types. Protein analysis revealed that non-defatted control flour and the 2-propanol (20 °C) defatted flour had similar protein compositions, whereas defatting with 2-propanol at 75 °C or WSB at 20 °C changed the protein composition by reducing the extractability of gliadins in 60% (v/v) ethanol, thus leading to increased glutenin content (Schaffarczyk et al., 2016a). The quantitation of the protein fractions as affected by the defatting method showed moderate differences, but in our view these changes in the protein composition could not explain that 2-propanol (75 °C) and WSB- (20 °C) defatted flour were not capable of forming a functional gluten network.

Activity-guided fractionation of wheat lipids

The separation of lipid mixtures into polar and non-polar lipids as well as into specific wheat lipid classes, followed by the determination of the relationships between specific wheat lipid classes and their functional effects in breadmaking, was the third aim of this work.

Initially, untreated dough lipids and lipase treated dough lipids were separated into a polar and non-polar fraction. Preliminary tests showed that the batch procedure according to Selmair (2010) and the solid phase extraction according to Bateman et al. (1997) provided inferior separation of non-polar and polar lipids than a modified batch procedure using silica gel as stationary phase and diethyl ether/petrol ether (90:10) and methanol/water (90:10) as extraction solvent (Ponte & De Stefanis, 1969). For further separation of polar lipids, size exclusion chromatography with Bio Beads S-X3 or Sephadex LH20 and different solvents and solvent mixtures (dichloromethane, methanol and hexane) was studied. The eluate was monitored by a RI-detector. Peaks were collected, the solvent was evaporated and the fractions were analyzed by the standard HPLC-ELSD method. However, neither for Bio Beads S-X3 nor for Sephadex LH20 a satisfactory separation of lipid classes could be achieved. It appears that the lipids formed micelles during the separation, which could not be

separated with any solvent combination. Therefore, a silica gel column chromatography with a gradient from 100% dichloromethane to 100% methanol was used to separate polar untreated and polar lipase-treated lipids. Sub-fractions enriched in specific lipid classes were combined. Six and eight polar lipid fractions were isolated from lipase-treated and untreated polar lipids, respectively. The lipid compositions of the combined fractions were quantitated by HPLC-ELSD. Different numbers of fractions were obtained with or without lipase because the enzyme changed the lipid composition of the polar fraction, and, for example, MGDG were not present any more in the lipase-treated lipids. The fractions (60 mg) were mixed with base lipids (60 mg flour lipids), analyzed for their lipid composition by HPLC-ELSD and studied for their functional effects (Schaffarczyk et al., 2016a).

Relationships between lipid mixtures and their functional effects in wheat breadmaking

Addition of lipid mixtures enriched in non-polar lipids caused a significant bread volume decrease with a tendency that non-polar lipase-treated lipids caused an even greater decrease of the bread volume than non-polar untreated lipids. Unsaturated FFA such as linoleic acid, which is the dominant FFA in wheat flour, are the main responsible lipid classes for poor loaf volume because they promote expanded monolayers leading to instability of the liquid lamellae. In contrary, addition of lipid mixtures enriched in polar lipids caused a significant bread volume increase due to the stabilization of gas cells. Polar lipase-treated lipids caused a significantly higher bread volume compared to polar untreated lipids. Further, all sub-fractions of polar lipase-treated dough lipids increased the bread volume compared to the control. The best functional effects were found for fractions containing MGMG, DGDG and NALPE as the main lipid classes (Schaffarczyk et al., 2016b). The results allowed the conclusion that an optimal baking lipase should preferably hydrolyze MGDG and NAPE. A complete degradation of DGDG can be considered unfavorable because the functional effects of DGDG are superior to those of DGMG.

The partial degradation of DGDG to a molar proportion of DGDG and DGMG of 1:1 provided one of the highest bread volumes, obviously due to synergistic effects. With increasing DGMG and decreasing of DGDG concentration the baking potential became lower. These synergistic effects between lipids are probably based on the indirect mechanism of lipids on the stabilization of the gas cell interface by lipid-lipid interactions. Synergistic effects between lipid classes could occur because monolayers consisting of a suitable mixture

of lipid classes with different mesomorphy types are similarly stable than monolayers of lipids of the lamellar type. Mixed micelles with different lipid classes reduce the energy barrier to adsorb to the interface and therefore increase the availability of surface-active lipids at the interface. Altogether, the most probable mode of action is the direct influence of the lipid classes on the gas cell interface through direct adsorption and formation of condensed monolayers. Our reconstitution baking tests confirmed the theoretical mechanism of baking lipases suggested by Gerits et al. (2014), since the lipase reaction products (MGDG → MGMG, NAPE→NALPE) mainly contributed to the bread volume increase due to preferential formation of the lamellar mesophase. Also synergistic effects need to be taken into consideration, as the formation of DGMG only has an improving effect in combination with other lipids promoting the lamellar or/and hexagonal phase I. This denotes the importance of the optimum lipid composition and the breakdown of lipids to obtain an increase of the bread volume.

In this context, lipid analysis showed that overdosing of lipase or long fermentation times caused a decrease of the bread volume due to extensive lipid hydrolysis by lyso-phospholipase and lyso-galactolipase activity of baking lipases. After a fermentation time of 60 min, the maximum concentration of beneficial lipase reaction products were reached for all lipases. For this fermentation time, an optimum baking performance was expected from the lipid profiles. The concentration of the reaction products MGMG and NALPE reached their maxima. After prolonged incubation the generated lyso-galactolipids and lyso-phospholipids were further hydrolyzed upon overproofing. The generation of MGMG by lipases is limited by the initial concentration of the educt MGDG in wheat flour. All examined lipases hydrolyzed MGDG almost completely yielding a maximum MGMG concentration after 60 - 80 min of fermentation. Therefore, the concentration of MGMG cannot be increased further with any baking lipase because the substrate is limited.

In order to increase the amount of MGDG in wheat flour by hydrolysis of DGDG, we examined the different α-galactosidases (α-(1,3/1,6)-galactosidase (New England BioLabs), recombinant E. coli α-(1,3/1,6)-galactosidase (R&D Systems), and α-galactosidase (Novozymes)). Theoretically, the increased amounts of MGDG would lead to high amounts of beneficial MGMG after subsequent incubation with lipases. However, DGDG was no substrate for any of the α-galactosidases neither in a dough system nor in a buffer system. Even the more polar lipase reaction product DGMG was not hydrolyzed by α-galactosidases.

It is questionable if other α-galactosidases with different substrate specificity would hydrolyze wheat flour lipids resulting in improved functional effects.

To get information if a certain amount of non-polar lipids in combination with polar lipids is required to achieve an optimal improver effect, the total amount of lipid added in the reconstitution baking test was kept constant at 120 mg/10 g flour, but the proportion of non-polar and polar lipids, either from untreated or lipase-treated dough or from flour was modified (Schaffarczyk et al., 2016b). In contrast to the reconstitution baking test with 50% base lipids and 50% polar lipids, 100% polar untreated dough lipids were more active compared to lipase-treated ones. The combination of 30 mg nonpolar (lipase-treated or untreated) dough lipids and 90 mg polar lipase-treated dough lipids tended to increase the bread volume compared to the polar lipids alone. It appears that the polar lipid reaction products of baking lipases need some background lipids or non-polar lipids to provide optimal functional effects. Figure 4.3 shows the specific bread volume of recombined flour with different combinations of base lipids (flour lipids) and polar lipase-treated dough lipids.

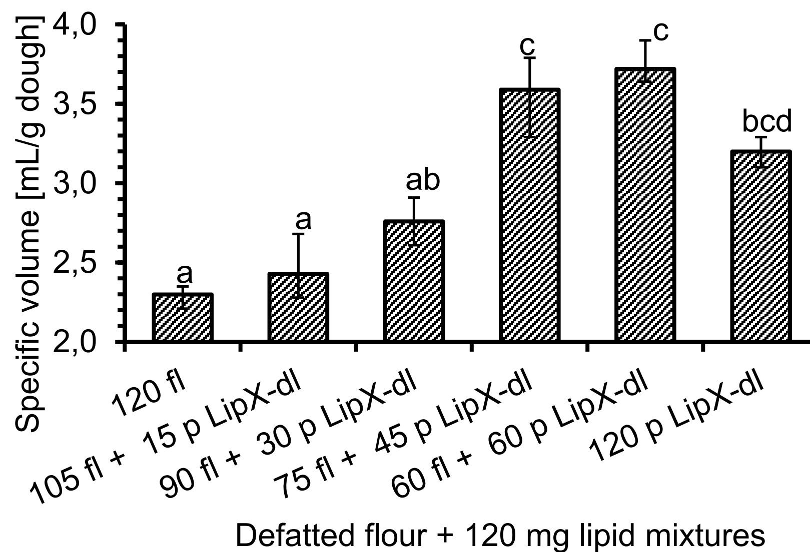

Figure 4.3: Specific volume of micro-breads as affected by the increasing proportion of polar lipase-treated lipids. Reconstituted flour was composed of 2-propanol defatted flour (9.50 g; 8.60 g dm) and 120 mg lipid mixtures covering a range between 100% flour lipids and 100% polar lipase-treated lipids (extracted with WSB, 20 °C). Error bars represent mean standard deviations of triplicate determinations. Mean values associated with different letters are significantly different (one-way ANOVA, Tukey test, $p \leq 0.05$). fl, flour lipids; LipX-dl, Lipopan Xtra-treated (130 mg/kg flour) dough lipids; np, non-polar; p, polar.

This experiment emphasized that 100% polar lipase-treated lipids were less active than in combination with 50% unmodified flour lipids. Further, the total amount of added lipid in the

reconstitution baking test was also modified. Loaf volume trends observed on incremental addition of different flour lipids, dough lipids or lipase-treated dough lipids (0 - 200 mg) showed significant differences within each lipid fraction (Schaffarczyk et al., 2016b). The incremental addition of polar lipase-treated lipids caused volume decrease to a minimum at 120 mg lipids/10g flour, whereas polar flour lipids and dough lipids already provided a bread volume increase from a concentration of 120 mg lipids/10 g flour. Overall, polar lipase-treated lipids were less active than polar untreated lipids. This experiment confirmed that in contrast to polar untreated lipids, polar lipase-reaction products need non-polar background lipids for maximal volume increase. Synergistic effects of the generated lipid classes with non-polar lipids might play a key role in the mechanism of baking lipases.

The various reconstitution baking tests with different fractions of lipid mixtures provided insights in the mechanism of baking lipases. However, the reasons for the synergistic effects of polar lipase-treated lipids and non-polar lipids are still not well understood. The credibility of the fractionation–reconstitution approach has been controversially discussed among scientists. One critical point is the impact of extraction solvents on flour constituents and functionality. As already descript above, the extraction solvents and extraction temperature can change the quantitative protein composition. Further, defatting followed by re-addition does not necessarily provide the lipids in their native state and location in the dough. It is in particular challenging to achieve a dispersion of the added lipids in the dough which is comparable to a dough from native flour. Against this background, we confirmed the suitability of the present baking system by quantitating the composition of free and bound lipids of doughs from recombined flours in comparison to lipids of doughs from native and lipase-treated flours. Free lipids of lyophilized recombined doughs were extracted through stirring with n-hexane. The remaining bound lipids were then extracted with WSB. The free lipids contained mainly TG, DG and FFA for doughs from both native and recombined flour. Looking at the bound lipids of dough from native flour in comparison to dough from recombined flour with 60 mg non-polar dough lipids + 60 mg polar dough lipids, and of dough from fermented flour (130 mg/kg Lipopan Xtra) in comparison to dough from recombined flour with 60 mg non-polar lipase-treated dough lipids + 60 mg polar lipase-treated dough lipids, no significantly differences could be observed. The lipid composition of recombined doughs with 120 mg polar dough lipids or 120 mg polar lipase-treated dough lipids differed only in those lipid classes that were hydrolyzed or generated by lipases, but not in the distribution of free and bound lipids. Thus, the lipid binding experiment did not provide

an explanation why polar dough lipids are more active than polar lipase-treated dough lipids. No significant differences in the behavior of lipids in recombined flour compared to native flour in regard to lipid binding was observed, confirming the suitability of the present baking system. This fractionation–reconstitution procedure is the only system for the examination of the relationship of lipid classes and their functional effects and, hence, also the only possibility for such studies up to date.

The fractionation–reconstitution approach can also be used for lipase activity assays to determine the substrate specificity in a dough system. Lipase activity assays against a standard substrate are commonly used for standardization of industrial lipase-products, although this determined activity does not necessarily reveal the real activity spectrum in the final application. As mentioned above, the hydrolytic profiles of lipases differ slightly depending on the assay system such as aqueous system, simulated dough-system with wheat starch, cellulose, SiO_2, glass beads, Al_2O_3 or real dough. Reconstitution baking test with specific lipid classes added to defatted flour, followed by HPLC quantitation of the hydrolysis after lipase incubation, would be a good assay to determine the real activity of a given lipase toward each lipid substrate in dough. However, preparation of the dough, freeze-drying and extraction of the lyophilized dough is very time consuming and, therefore, not practicable for routine analysis. To screen different lipases for their activity toward specific lipid classes, for example high activity toward NAPE, we started to establish an activity assay for baking lipases in a buffered system. Specific lipid classes and the internal standard cholesterol were emulsified in phosphate buffer (pH 5.54, 0.1 mol/L) and 0.15% Brij 35. After incubation, the lipids were extracted with dichloromethane and analyzed by HPLC. In general, the assay worked, but due to the high standard deviations, the obtained specific activities can only be seen as an approximate value. Further work is needed to optimize the system to improve the stability of the emulsion and the reproducibility, e.g. by studying different proportions of buffer, emulsifier and lipids or by studying different emulsifiers. A direct quantitation of the released FFA using a colorimetric enzyme kit could also be a possibility to determine the activity of lipases toward specific lipid classes without extracting the lipase reaction products from the buffered system.

Based on the results gained in this study, the theoretical mechanism of baking lipases, in terms of formation of stable interfacial lipid layers, was confirmed. We speculate that the ‘perfect’ baking lipase preferably hydrolyses MGDG and NAPE, but has only moderate activity toward DGDG. The lipase reaction products (MGDG → MGMG, NAPE→NALPE)

mainly contributed to the bread volume increase due to preferential formation of the lamellar mesophase. We support the hypothesis of Gerits et al. (2014) that lipases catalyze the transition of lipid classes promoting the detrimental hexagonal II mesophase into lipid classes promoting the lamellar and hexagonal I mesophases, which are considered to be highly effective in stabilizing gas cells. A factor that should not be underestimated is the synergistic effect between lipids based on the indirect mechanism of lipids on the stabilization of the gas cell interface. For optimal performance, the complementary effect of stable monolayers formed by a mixture of lipids favoring the hexagonal I and hexagonal II mesophases is needed to guarantee gas cell stability. Furthermore, the emulsification of non-polar oil droplets hinders their adsorption at the gas cell interface, thereby stabilizing the condensed monolayers of surface-active lipid classes, in other words, non-polar lipids should be kept away from gas cell interfaces. We assume that the polar reaction products of baking lipases need the presence of unmodified base lipids or non-polar lipids to provide the best functional effects by the complementary effect of stable monolayers. However, further studies are needed to confirm this hypothesis and to understand the synergistic effects of polar lipase-treated lipids and non-polar lipids. Further, it remains unclear whether altering the polarity of the endogenous lipids has impact on gluten protein polymerization during baking. Further research is needed in this area.

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5. Future research

Future research on lipases and lipids in wheat breadmaking can be subdivided into several different topics. With respect to lipid analysis, further studies should focus on developing a quantitative liquid chromatography–mass spectrometry (LC-MS) method for wheat flour lipids and lipase reaction products based on the good separation of lipid classes obtained with the established HPLC-ELSD method. For a reproducible and highly sensitive lipid profiling method for all types of lipids it is necessary to provide a good LC separation. Additionally, the analysis should allow accurate identification of lipid molecular species and the determination of fatty acid chain compositions. Moreover, a high-throughput and accurate identification of lipid molecular species should be ensured. With structural analysis and quantitation of even low-abundance compounds, the lipid profiling technique would provide new insight into the relationship of endogenous wheat flour lipid species and end-use functionality.

Secondly, this study demonstrated the successful implementation of the fractionation–reconstitution approach to determine the functional effects of specific lipid classes. However, there is also need for further work concerning the synergistic effects of specific lipid classes and, in particular, of polar lipase-treated lipids and non-polar lipids. The understanding of the mechanism of action of baking lipases is essential for optimizing and expanding the field of application. A profitable field for expansion of lipase application would be fine bakery products. Up to date, baking lipases are only used in combination with other enzymes for bread production. In fine bakery products, in particular products with relatively large amounts of bakery or milk fat, lipase application may lead to a rancid off-flavor. Due to the hydrolysis of TG by baking lipases, the release of butyric acid limits such applications. Screening for proper (phospho)lipases would allow lipase application to improve the quality of fine bakery products. Therefore, future studies will, for example, have to characterize the off-flavor by methods of molecular sensory science, such as gas-chromatography olfactometry.

Thirdly, the development of an enzyme activity assay toward specific lipid classes for routine analysis would provide the possibility to screen for new lipases with specific, optimized hydrolytic profiles.

It is clear, that some challenges still need to be addressed, but the results of this study have shown a great potential for future research.

6. Summary

Beside synthetic emulsifiers, such as diacetyl tartaric esters of monoglycerides, lipases along with other enzymes are being used to improve the baking performance of wheat flours or to compensate for variations in quality of wheat flour. Selective modification of the wheat lipid composition by lipases drastically impacts the baking performance of the flour and the end-product quality. Currently available lipases for baking applications hydrolyze a number of lipid structures in flour and lead to improved surface-activity of endogenous lipids, thus resulting in significant increase in bread oven rise and specific volume. However, in-depth understanding of lipase-mediated reaction products is missing and especially information regarding the functional effects of lipid classes generated by the action of lipases is still very scarce. A better characterization of these lipase reaction products would be an important step in understanding the mechanism of lipases in baking applications and, thus, in developing improved baking lipases. Therefore, the aim of this work was to determine the relationships between specific wheat lipid classes and their functional effects in breadmaking with regard to the optimal hydrolytic profile of lipases in relation to baking.

In the first part of this work a high-performance liquid-chromatography method with evaporative light scattering detection was established, which was able to separate and quantitate all wheat lipid classes and lipase reaction products. For identification and quantitation of the various lipid classes, several pure glycolipids and phospholipids were isolated from wheat lipids by a silica gel batch procedure and silica gel column chromatography. For quantitation of the lipid classes, cholesterol was used as internal standard. Wheat lipids, dough lipids and dough lipids after lipase addition were isolated by extraction with water-saturated *n*-butanol (WSB) (20 °C) and 2-propanol (75 °C). It was clearly shown that the former was the preferred solvent for lipid isolation. Moreover, the lipid composition after incubation with different concentrations of four baking lipases and increasing fermentation times (0-120 min) were quantitated. The data showed that specific lipid classes were hydrolyzed and the concentration of the corresponding reaction products increased. Especially digalactosyl diglycerides (DGDG) (-0.9 mmol/kg flour), monogalactosyl diglycerides (MGDG) (-0.4 mmol/kg) and N-acyl-phosphatidyl ethanolamine (NAPE) (-0.3 mmol/kg) were hydrolyzed and a concomitant formation of digalactosyl monoglycerides (DGMG) (+0.6 mmol/kg), monogalactosyl monoglycerides (MGMG) (+0.6 mmol/kg), and N-acyl-lysophosphatidyl ethanolamine (NALPE) (+0.5 mmol/kg) was found. After prolonged incubation or increased lipase concentration the

generated lyso-galactolipids and lyso-phospholipids were further hydrolyzed. Micro-baking tests on a 10 g flour basis showed bread volume increases up to 56-58% depending on the type and concentration of the added lipase. Beside the incubation time, the lipase concentration was also of major importance for the optimal lipid composition and the breakdown of lipids to obtain bread volume increase.

The second part focused on solving the core prerequisite of this work, the establishment of a micro-reconstitution baking test that provides the same functionality as native wheat flour and allows studying the functional effects of any type of lipid in breadmaking, including flour and dough lipids from wheat, lipase-modified wheat lipids, unmodified or modified wheat lipid fractions, individual lipid classes, and even non-wheat lipids. Wheat flour defatted with 2-propanol at 20 °C and WSB-isolated wheat lipids are suitable for this approach. Other defatting methods using 2-propanol under heating (75 °C) or WSB at 20 °C lead to a partial or complete loss of flour functionality in baking applications. Quantitation of gluten proteins showed that defatting with WSB (20 °C) or 2-propanol (75 °C) decreased the gliadin and increased the glutenin content. Possible reasons were thiol-disulfide interchange reactions either caused by heat (2-propanol, 75 °C) or by the solvent WSB, which affected gluten proteins. Further, confocal laser scanning microscopy showed that regular, interconnected gluten structures were only present in dough from flour defatted with 2-propanol at 20 °C. Dough made from flour defatted with the other solvents showed no protein network formation. Only precipitated proteins were present which were scattered and clustered, but not interconnected.

In the last part of the work the relationships between specific wheat lipid classes and their functional effects in breadmaking was revealed by means of activity-guided lipid fractionation of lipase-treated lipids and micro-scale reconstitution baking tests. The evaluation of the micro-scale reconstitution baking tests conducted for fractions of lipase-treated and untreated lipids showed that polar lipase-reaction products were responsible for their positive functional effects in wheat breadmaking. The bread volume increase was even higher for polar lipase-treated lipids than for polar untreated lipids. The polar fractions of lipase-treated and untreated dough lipids were further fractionated by column chromatography providing six and eight sub-fractions enriched in specific lipid classes, respectively. Fundamental knowledge about the influence of the major lipase reaction products on the overall baking performance was gained. The negative influence of the free fatty acids was counteracted by the highly positive influence of all fractions from polar lipase-treated dough

lipids. The best functional effects were found for fractions containing MGMG, DGDG and NALPE as the main lipid classes. The results allowed the conclusion that an optimal baking lipase should preferably hydrolyze MGDG and NAPE. Galactolipids with one galactose and one monoacyl moiety, like MGMG, possess by far better hydrophilic lipophilic balance in comparison with the diacyl-compound MGDG. This fact demonstrates the importance of an appropriate ratio between hydrophilic and lipophilic moieties in the molecular structure of each individual surfactant for its functional effects. Synergistic effects of DGDG and their products DGMG (molar proportion 1:1) provide even a greater bread volume increase than DGDG alone. These synergistic effects between lipids are probably based on the indirect mechanism of lipids on the stabilization of the gas cell interface. DGMG only have an improving effect in combination with other lipids promoting the lamellar or/and hexagonal phase I. This denotes the importance of the optimum lipid composition after breakdown of native lipids to obtain an increase of the bread volume. The most probable mode of action is the direct influence of the lipid classes on the gas cell interface through direct adsorption and formation of condensed monolayers. Our reconstitution baking tests confirmed the theoretical mechanism of baking lipases, since the lipase reaction products favoring the formation of the lamellar mesophase mainly contributed to the bread volume increase.

Reconstitution baking (defatted flour plus lipid or lipid fraction added) partly provided contradictory results. In contrast to polar untreated lipids, polar lipase-reaction products need non-polar background lipids for maximal volume increase. Synergistic effects of the generated lipid classes with non-polar lipids might play a key role in the mechanism of baking lipases. It appears that generated lipid classes promoting the hexagonal I mesophase (DGMG, LPC) give expanded monolayers leading to instability of the liquid lamellae. For optimal performance, the complementary effect of stable monolayers formed by a mixture of lipids favoring the hexagonal I and hexagonal II mesophases is needed to guarantee gas cell stability. Furthermore, the emulsification of non-polar oil droplets hinders their adsorption at the gas cell interface, thereby stabilizing the condensed monolayers of surface-active lipid classes.

Although these experiments have advanced the knowledge on the functional effects of lipase-treated lipids, in particular the reasons for the synergistic effects of polar lipase-treated lipids and non-polar lipids need further attention.

7. Zusammenfassung

Neben synthetischen Emulgatoren, wie Monoacetyl- und Diacetylweinsäureestern von Mono- und Diglyceriden, werden Lipasen und andere Enzyme bei der Herstellung von Backwaren verwendet, um die Backeigenschaften von Weizenmehlen zu verbessern oder schwankende Mehlqualitäten auszugleichen. Die gezielte Veränderung der Zusammensetzung von Weizenlipiden durch Lipasen hat einen großen Einfluss auf die Backeigenschaften des Mehles und die Qualität des Endproduktes. Lipasen für Backanwendungen hydrolysieren eine Reihe mehleigener Lipidklassen und erhöhen dadurch den Anteil polarer Lipide im Teig, wodurch die mehleigenen Lipide als „endogene“ Emulgatoren wirken und die Teig- und Backeigenschaften verbessern. Bisher fehlen jedoch grundlegende Erkenntnisse über die Reaktionsprodukte von Weizenlipiden mit Lipasen, und es fehlen Informationen über deren funktionelle Wirkungen. Die sorgfältige Charakterisierung dieser Reaktionsprodukte ist daher eine Voraussetzung zum besseren Verständnis der Wirkung von Lipasen für Backanwendungen und ermöglicht die Entwicklung optimierter Backlipasen. Das Ziel dieser Arbeit war deshalb die Untersuchung der Zusammenhänge zwischen einzelnen Weizenlipidklassen und deren funktionellen Wirkungen bei der Herstellung von Backwaren. Dazu sollten endogene Weizenlipide durch den Zusatz von Backlipasen teilweise hydrolysiert und die Wirkungen der unmodifizierten und modifizierten Lipidgemische verglichen werden.

Im ersten Teil der Arbeit wurde eine verbesserte Hochleistungsflüssigkeitschromatographie-Methode mit Lichtstreuungsdetektion (HPLC-ELSD) entwickelt, welche es ermöglicht, alle Weizenlipidklassen und Reaktionsprodukte der Lipasen zu trennen und zu quantifizieren. Zur Identifizierung und Quantifizierung der verschiedenen Lipidklassen wurden zahlreiche reine Glykolipide und Phospholipide als Referenzsubstanzen aus Weizenlipiden mittels eines Kieselgel-Batchverfahrens und einer Kieselgel-Säulenchromatographie isoliert. Cholesterin wurde als interner Standard zur Quantifizierung der Lipidklassen verwendet. Weizenlipide, Teiglipide und lipasebehandelte Teiglipide wurden durch Extraktion mit wassergesättigtem *n*-Butanol (WSB) (20 °C) und 2-Propanol (75 °C) isoliert. Es wurde gezeigt, dass WSB das bevorzugte Extraktionsmittel zur Isolierung von Weizenlipiden ist. Des Weiteren wurde die Lipidzusammensetzung nach Inkubation von Teigen in Abhängigkeit von verschiedenen Lipasen, von der Lipasekonzentrationen und von der Fermentationszeit (0-120 min) quantifiziert. Die Auswertung zeigte, dass spezifische Lipidklassen hydrolysiert wurden und die Konzentration der entsprechenden Reaktionsprodukte zunahm. Insbesondere wurden Digalactosyldiglyceride (DGDG) (-0.9 mmol/kg), Monogalactosyldiglyceride (MGDG) (-0.4

mmol/kg), N-Acylphosphatidylethanolamin (NAPE) (-0.3 mmol/kg) hydrolysiert und gleichzeitig Digalalctosylmonoaglyceride (DGMG) (+0.6 mmol/kg), Monogalactosylmonoglyceride (MGMG) (+0.6 mmol/kg) und N-Acyllysophosphatidylethanolamin (NALPE) (+0.5 mmol/kg) gebildet. Nach verlängerter Inkubationszeit und bei erhöhter Lipasekonzentration wurden die gebildeten Lysogalactolipide und Lysophospholipide weiter hydrolysiert. Mikro-Backversuche mit 10 g Mehl zeigten eine Zunahme des Brotvolumens von bis zu 58 %, abhängig von der eingesetzten Lipase und deren Konzentration. Neben der Fermentationszeit war die Lipasekonzentration ein wichtiger Parameter zur Beeinflussung der Lipidzusammensetzung und der Erhöhung des Brotvolumens, d. h. jede Lipase wies ein spezifisches Konzentrationsoptimum auf, bei dem das höchste Brotvolumen erzielt wurde.

Der zweite Teil der Arbeit konzentrierte sich auf die Entwicklung eines Mikro-Rekonstitutionsbackversuches, der es ermöglichte, die funktionellen Wirkungen von Mehl- und Teiglipiden aus Weizen, von lipasebehandelten Weizenlipiden, von behandelten und unbehandelten Weizenlipidfraktionen, von individuellen Lipidklassen und von Lipiden anderer Herkunft, zu untersuchen. Dafür waren mit 2-Propanol bei 20 °C entfettetes Weizenmehl als Basis und mit WSB-isolierte Weizenlipide als Zusatz geeignet. Andere Entfettungsmethoden für das Basismehl, z. B. mit 2-Propanol in der Hitze (75 °C) oder mit WSB bei 20 °C, führten zum teilweisen oder vollständigen Verlust der Mehlfunktionalität für Backanwendungen. Die Quantifizierung der Glutenproteine in entfettetem und nicht entfettetem Mehl zeigte, dass die Entfettung mit WSB (20 °C) oder 2-Propanol (75 °C) den Gliadingehalt verringerte und den Gluteningehalt erhöhte. Möglicherweise waren Gliadine über einen hitze- (2-Propanol, 75 °C) oder lösungsmittelinduzierten (WSB) Thiol-Disulfid-Austausch an die Glutenine gebunden worden. Konfokale Laser-Scanning-Mikroskopie der Teige zeigte, dass nur in dem Teig, welcher mit 2-Propanol bei 20 °C entfettet wurde, eine vernetzte Glutenstruktur vorlag. In Teigen aus Mehlen, welche mit den anderen Methoden entfettet worden waren, lag kein Proteinnetzwerk vor, sondern nur verstreute, nicht miteinander verbundene Proteinpartikel.

Im letzten Teil der Arbeit wurden die Beziehungen zwischen einzelnen Lipidklassen und deren funktionellen Wirkungen in Backwaren durch aktivitätsorientierte Lipidfraktionierung von lipasebehandelten Lipiden und durch Mikro-Rekonstitutionsbackversuche aufgezeigt. Mikro-Rekonstitutionsbackversuche mit Fraktionen von lipasebehandelten und unbehandelten Weizenlipiden zeigten, dass die polaren Reaktionsprodukte für die positiven funktionellen Wirkungen bei der Brotherstellung verantwortlich waren. Die Zunahme des Brotvolumens

war für behandelte polare Lipide größer als für unbehandelte polare Lipide. Die polaren Fraktionen von lipasebehandelten und unbehandelten Lipiden wurden mit Hilfe einer Säulenchromatographie weiter aufgetrennt, so dass sechs bzw. acht Fraktionen erhalten wurden, in denen einzelne Lipidklassen angereichert waren. Fraktionen, die hauptsächlich MGMG, DGDG und NALPE enthielten, führten zur stärksten Erhöhung des Brotvolumens. Aus den Ergebnissen kann abgeleitet werden, dass eine optimale Backlipase bevorzugt MGDG und NAPE hydroylsiert. Galactolipide mit einem Galactose- und einem Fettsäurerest, wie z. B. MGMG, haben im Vergleich zur Diacyl-Verbindung MGDG ein günstigeres Verhältnis hydrophiler und lipophiler Strukturelemente. Dadurch wird die Bedeutung eines geeigneten Verhältnisses von hydrophilen und lipophilen Gruppen in der Struktur jedes oberflächenaktiven Moleküls für seine funktionellen Wirkungen ersichtlich. Ein Gemisch aus DGDG und deren Hydrolyseprodukt DGMG (molares Verhältnis 1:1) lieferten durch synergistische Effekte sogar größere Volumenzunahmen als DGDG alleine. Diese synergistischen Effekte zwischen verschiedenen Lipidklassen sind wahrscheinlich auf die indirekte Stabilisierung der Grenzflächen durch Lipide zurückzuführen. DGMG hat nur in Kombination mit Lipiden, die eine lamellare Phase und/oder eine hexagonale Phase I ausbilden, eine positive funktionelle Wirkung. Deshalb ist die optimale Lipidzusammensetzung durch den lipaseinduzierten Abbau von Lipiden essentiell für eine Zunahme des Brotvolumens. Die positive Wirkung von Backlipasen kann durch die direkte Adsorption gebildeter Lipidklassen an die Grenzflächen der Gasblasen im Teig und die Bildung von komprimierten Monoschichten erklärt werden. Die Backversuche bestätigten diese Hypothese, da die Reaktionsprodukte der Lipasen bevorzugt lamellare Mesophasen ausbildeten und so zur Volumenvergrößerung betrugen.

Weitere Rekonstitutionsbackversuche mit entfettetem Mehl, denen Lipide oder Lipidfraktionen zugesetzt wurden, ergaben teilweise widersprüchliche Ergebnisse. Im Gegensatz zu polaren unbehandelten Lipiden, brauchten polare Reaktionsprodukte von Lipasen offenbar unpolare Basislipide, um eine maximale Volumenzunahme zu erreichen. Wahrscheinlich spielten auch hier synergistische Effekte der gebildeten Lipidklassen mit unpolaren Lipiden eine wichtige Rolle für die Funktionalität von Backlipasen. Gebildete Lipidklassen, die eine hexgonale Mesophase I ausbilden (DGMG, LPC), destabilisieren die Grenzflächen durch Bildung expandierter Monoschichten. Zur Stabilisierung der Grenzflächen ist eine Mischung von Lipiden nötig, die eine hexagonale Mesophase I und II ausbilden und durch komplementäre Effekte eine stabile Monoschicht aufbauen können.

Außerdem verhindert die Emulgierung unpolarer Öltröpfchen deren Adsorption an die Grenzfläche, wodurch die kondensierte Monoschicht aus grenzflächenaktiven Lipidklassen stabilisiert bzw. nicht geschwächt wird.

Die vorliegende Arbeit hat das Wissen über die funktionellen Wirkungen von lipasebehandelten Lipiden deutlich erweitert. Die den synergistischen Effekten von Gemischen aus polaren lipasebehandelten und unpolaren Lipiden zugrundeliegenden Mechanismen müssen in weiterführenden Arbeiten aufgeklärt werden.