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Hydrolysis of gluten and the formation of flavor precursors during sourdough fermentation

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Abbreviations

AP	Aminopeptidases
AU	Absorption unit
B.	Bacillus
BC	before christ
C.	Candida
cfu	Cell forming unit
CIEX	Cation exchange chromatography
D	Dough
D/TP	Di- and tripeptidase
Da	Dalton
DTT	Dithiothreitol
EP	Endopeptidases
FD	Flavor dilution
FITC	Fluorescenceisothiocyanate
GMP	Gluten macropolymer
GPC	Gelpermeation chromatography
GSH	Glutathione
HMW	High molecular weight
HPLC	High performance liquid chromatography
IEX	Ion exhange chromatography
IPAD	Integrated pulsed amperometric detection
LAB	Lactic acid bacteria
L.	Lactobacillus
Lc.	Lactococcus
Leuc.	Leuconostoc

LMW	Low molecular weight
LTH	Lebensmitteltechnologie Hohenheim
M	Molar
MALLS	Multiangle laserlight scattering
MW	Molecular weight
n.g.	Not grown
OD	Optical density
PAGE	Polyacrylamid gelelectrophoresis
PP	Prolinepeptidase
rfu	Relative fluorescence unit
RP	Reversed phase
S.	Saccharomyces
SDS	Sodium dodecylsulfate
SEC	Size exclusion chromatography
SPE	Solid phase extraction
TFA	Trifluoracetic acid
TMW	Technische Mikrobiologie Weihenstephan

1. Introduction 7

1.1 Technology of sourdough 7

1.2 Microflora of sourdough fermentations....8

1.3 Flavor of wheat bread....9

1.4 Proteolytic activity and amino nitrogen demand of lactic acid bacteria....13

1.5 Wheat proteins and gluten macropolymer....16

1.6 Determination of amino acids in complex media by anion exchange chromatography and integrated pulsed amperometric detection 17

1.7 Aim of this work 18

2. Materials and Methods....20

2.1 General Methods....20

2.1.1 Strains and culture conditions....20

2.1.2 Media 20

2.1.3 Determination of viable cell counts, and pH. 21

2.1.4 Determination of carbohydrates and organic acids in dough. 21

2.1.5 Determination of total amino nitrogen content....21

2.1.6 Size exclusion chromatography (SEC) of SDS-soluble proteins and SDS-DTT soluble proteins. 22

2.1.7 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)....23

2.2 Generation of flavor precursor compounds during sourdough fermentation....23

2.2.1 Sourdough fermentation....23

2.2.2 Baking experiments. 24

2.2.3 Amino acid analysis by post column derivatization 26

2.3 Screening for proteolytic active microorganisms....27

2.3.1 Strains and culture conditions....27

2.3.2 Media 27

2.3.3 Screening on agar plates....28

2.3.4 Determination of proteolytic activity by fluorescent substrates 28

2.4 Nitrogen demand and peptide utilization by L. sanfranciscensis 29
2.4.1 Amino nitrogen free media 29
2.4.3 Doughs for peptide extraction 30
2.4.4 Preparative size exclusion chromatography 30
2.4.5 Cation exchange chromatography 31
2.4.6 Reverse-phase chromatography 31
2.4.7 Growth experiments with dough fractions 32
2.5 De-/polymerization and proteolysis of gluten proteins in sourdough 32
2.5.1 Sourdough fermentation 32
2.5.2 Extraction of doughs and reversed-phase (RP)-HPLC analysis of dough extracts. 33
2.6 De-/Polymerization and proteolysis determined with FITC labeled wheat proteins 34
2.6.1 Extraction of wheat proteins. 34
2.6.2 Labeling of wheat proteins with FITC. 34
2.6.3 Purification of labeled proteins. 34
2.6.4 Reconstitution of wheat doughs with fluorescent labeled proteins and dough fermentation 35
2.7 Amino Acid Analysis from Food by IPAD 36
2.7.1 Chemicals and reagents 36
2.7.2 Liquid chromatography 37
2.7.3 Solid Phase Extraction 39
2.7.4 Recovery of amino acids from food samples. 40
3. Results 42
3.1 Contribution of microorganisms and wheat enzymes on the flavor of rolls 42
3.1.1 Effect of salt, acid and dithiothreitol (DTT) on the total amino nitrogen content in sterile wheat doughs. 42
3.1.2 Total amino nitrogen content in fermented wheat doughs 43
3.1.3 Changes in individual amino acid concentration 46
3.1.4 Sensory evaluation of rolls 49
3.2 Screening of proteolytic active lactic acid bacteria 50
3.3 Nitrogen demand and peptide utilization by *L. sanfranciscensis* 56
3.3.1 Dough extraction and preparative SEC 56
3.3.2 Dough fractionation with cation exchange chromatography 59
3.3.3 Fractionation by reversed phase chromatography 61

3.4 Depolymerization and proteolytic breakdown during sourdough fermentation 63
3.4.1 Microbial growth and proteolysis during dough fermentation. 64
3.4.2 Quantification and size distribution of SDS-soluble proteins. 65
3.4.3 Sequential extraction of gluten proteins and qualitative and quantitative analysis by SDS-PAGE and RP-HPLC. 69
3.5 Depolymerization and proteolytic breakdown of FTC-Proteins 74
3.5.1 Sequential extraction, labeling and purification of wheat proteins. 74
3.5.2 Correlation of protein size and elution volume of SEC 76
3.5.3 Influence of modified proteins and protease on dough fermentation.............................76
3.5.4 Proteolytic degradation of FITC-proteins 77
3.5.5 Incorporation of proteins into the gluten macropolymer...80
3.6 Amino acid determination by IPAD...83
3.6.1 Absorption of amino acids and removal of sugars..83
3.6.2 Influence of salt and pH on the elution of amino acids...85
3.6.3 Detection of sugars and amino acids by IPAD 88
3.6.4 Validation of the sample preparation with food samples 88
4. Discussion 93
4.1 Overview 93
4.2 Generation of flavor precursor compounds during sourdough fermentation............................94
4.3 Screening of proteolytic lactic acid bacteria..97
4.4 Nitrogen demand and peptide utilization by *L. sanfranciscensis*...98
4.5 Depolymerization and proteolytic breakdown during sourdough fermentation 100
4.6 Depolymerization and proteolytic breakdown of FITC-Proteins...103
4.7 Amino acid determination by IPAD...107
5. Conclusions 108
6. Zusammenfassung 110
7. References 112
8. Appendix 127

1. Introduction

1.1 Technology of sourdough

For at least 12.000 years cereals are a important component of human nutrition exemplified by excavation of grinding tools in the area of todays Syria (Katz and Voigt, 1986). At those times, cereals were consumed raw, as porridge or gruel. It is assumed that fermentative products for example beer or sour dough initially happened accidentally and without prior knowledge about the underlying processes. About 7000 to 6000 BC humans baked their own bread (Lönner and Ahrne, 1995). Excavations in Switzerland established that sourdough bread was part of the typical diet over 5000 years ago (Währen, 1985). According to Steinkraus (1983) the tradition of fermenting and baking cereals to obtain leavened bread with improved organoleptic properties is important all over the world, for example in Sudan (Kisra, prepared from Sorghum), India (Idli, prepared from rice, beans or chick peas), Mexico (Pozol, prepared from maize) and Europe (Sourdough, prepared from wheat or rye).

For rye bread acidification is of prime importance to achieve a raised bread because bread texture is mainly determined by carbohydrates like starch and pentosans. During dough preparing and baking endogenic enzymes like alpha-amylases degrade this matrix and gas generated by microbial metabolism can not be retained within the loaf. The result is a flat and hard “brick”. As alpha-amylases are acid intolerant, an acidification of dough inactivates these enzymes. The carbohydrate structure is not degraded and a spongy, aromatic bread is the result. Therefore rye sourdough fermentations have been performed, investigated and optimized for a long time.

Wheat bread does not necessarily need a pre-fermentation, because bread structure is determined to a great extent by gluten proteins. Evidence for the impact of specific metabolic activities on bread quality was provided concerning the production of antifungal and antibacterial metabolites during sourdough fermentation (Gänzle and Vogel, 2003, Lavermicocca et al., 2000), and the generation of flavor precursors and flavor volatiles (Hansen and Hansen, 1994; Thiele et al., 2002). The formation of expolysaccharides in dough improves wheat bread texture (Korakli et al., 2001, Brandt, 2001). The optimization of sourdough process for industrial applications in wheat baking requires insight into biochemical mechanisms responsible for the quality of sourdough bread.

Beneficial effects of sourdough fermentation on bread quality include, but are not limited to, a prolonged shelf life through inhibition of mould growth (Lavermicocca et al., 2000), increased loaf volume (Collar Esteve et al., 1994; Hansen and Hansen, 1996), delayed staling (Armero and Collar, 1998, Corsetti et al., 2000), improved bread flavor (Thiele et al., 2002), and an improved nutritional quality based on a lowered glycemic index (Liljeberg and Björck, 1996).

1.2 Microflora of sourdough fermentations

According to the “Leitsätze des Deutschen Lebensmittelbuchs” (Anonymous, 1994) sourdough is a dough which contains active or viable microorganisms for example lactic acid bacteria and yeasts. The continuous propagation of sourdough by back-slopping is leading to a stable microflora, characterized by a high acid tolerance and a metabolism well adapted to the cereal environment. This microflora is dominated by members of the genus *Lactobacillus* (Hammes and Vogel, 1997) mainly constituting heterofermentative species (Stolz, 1995). They can be allotted to the species *L. pontis* (Vogel et al., 1994; Müller et al., 2001) , *L. sanfranciscensis* (Kline and Sugihara, 1971; Weiss and Schillinger, 1984; Böcker et al., 1990), *L. fermentum*, *L. reuteri* (Vogel et al., 1994: Stolz et al., 1995: Hamad et al., 1997), and *L. panis* (Wiese et. al., 1996). The dominance of heterofermentative lactic acid bacteria can be explained by their effective maltose metabolism, by their capability to use fructose as electron acceptor, and their glucose accumulation. Additionally some other lactic acid bacteria are worth to mention, *L. amylovorus* (Vogel et al., 1996; Suwelack et al., 1997; Müller et al., 2001; Müller et al., 2000) and *Weissella confusa* (Vogel et al., 1996; Corsetti et al., 2001) constitute a dominant element in long term fermentations.

The sourdough flora is not only well characterized with respect to lactic acid bacteria, but also for yeasts data are available. The occurrence of *S. cerevisiae* is very often mentioned (Gobbetti, 1994; Ottogalli et al., 1996; Rossi, 1996) whereas its overall presence could be the result of its extensive use in bakeries. *Pichia membranaefaciens* (Rossi, 1996; Almeida and Pais, 1996; Paramithiotis et al., 2000) and *Candida milleri* synonymous to *C. humilis* (Yarrow, 1978; Spicher, 1987; Böcker 1993)

were also frequently described. In French wheat sourdough *Candida krusei* was the dominant yeast species (Brandt, 2001).

The microflora of sourdough strongly depends on the fermentation conditions. Fermentation temperature, dough yield, redox potential, fermentation time, manner of refreshment and last but not least the mother sponge are exhibiting strong influence on the microflora. Basically three types of sourdough fermentations can be distinguished (Böcker et al., 1995). The microflora of sourdoughs sustained by repeated inoculation at ambient temperature consists mainly of strains of *L. sanfranciscensis* (type I doughs). Most traditional sourdoughs can be classified as type I. Strains of *L. reuteri*, *L. fermentum*, *L. pontis*, *L. amylovorus* and *L. panis* are most frequently isolated from sourdoughs with longer fermentation times, or those doughs fermented at elevated temperature (type II doughs, Vogel et al., 1999). Type III doughs are artificial doughs initiated as souring enhancer by defined starter cultures for example *L. plantarum*, *L. brevis* and *Pediococcus pentosaceus* (Hochstrasser et al., 1993; Böcker et al., 1995). Additionally another fermentation type often used for wheat bead (Ciabatta, Baguette, Soda-Cracker) shall be mentioned. In these "type 0" doughs the preferment is inoculated only with bakers yeast and the fermentation time is between 3 and 24 hours. In addition to *S. cerevisiae* a variety of possibly important contaminants like *Candida crusei*, *L. plantarum*, *Leuconostoc mesenteroides*, and *Pediococcus pentosaceus* is found in these doughs (Brandt, 2001).

1.3 Flavor of wheat bread

Bread is prepared out of the essentially tasteless ingredients flour, water, salt and yeast. Almost all flavor active components are formed during dough fermentation and baking. In previous studies the aroma compounds contributing to the typical wheat bread flavor were identified (Schieberle, 1996) and are shown in Table 1.1.

Table 1.1: Key odorants showing high odor activities (Flavor Dilution (FD) factors) in wheat bread crust and crumb (Schieberle, 1996)

Odorant in	**Precursor**	**Smelling**	**FD factor** [a)]
Wheat bread crust			
2-Acetyl-1-pyrroline	Ornithine, proline	Roasty, cracker-like	512
4-Hydroxy-2,5-dimethyl-3(2H)-furanone	Fructose	Caramel-like	512
(E)-2-Nonenal	Lipids	Fatty	256
3-Methylbutanal	Leucine	Malty	128
3-Methylbutanoic acid	Leucine	Sweaty	128
2,3-Butandione		Buttery	64
3-Methylbutanol	Leucine	Fermentation-like	64
Wheat bread crumb			
(E,E)-2,4-Decadienal	Lipids	Fatty, waxy	512
(E)-2-Nonenal	Lipids	Fatty	512
4,5-Epoxy-(E)-2-decenal	Lipids	Metallic	512
3-Methylbutanol	Leucine	Fermentation-like	256
2-Phenylethanol	Phenylalanine	Flowery	256
1-Octen-3-one	Lipids	Mushroom-like	128
2,3-Butandione		Buttery	128
3-(Methylthio)propanal	Methionine	Cooked potato	128
3-Methylbutanoic acid	Leucine	Sweaty	128

a) The FD factor is calculated by the stepwise dilution of extract with solvent, separation by high resolution gas chromatography and, at the given retention indices, a check by the assessor whether the respective odor quality is yet detectable or not.

The roasty aroma of wheat bread depends on the formation in the crust of flavor active compounds during the baking process. 2-Acetyl-1-pyrroline was identified as the character impact compound for the odor of wheat bread crust, its precursors are ornithine and proline (Schieberle, 1990). In addition 11 more volatile compounds are important for the crust and crumb aroma (Schieberle, 1996). These compounds originate from the fatty acid oxidation (nonenal or decadienal), the thermal degradation of sugars (4-Hydroxy-2,5-dimethyl-3(2H)-furanone), the thermal degradation of amino acids

(Methylbutanal, methional and acetylpyrroline), or they are products of microbial metabolism of amino acids (methylbutanol, 3-(methylthio)-propan-1-ol or phenylethanol). It was already shown, that amino acids present in dough are reduced to 10 – 20% of their initial value during baking of sourdough breads (Gobbetti et al., 1995).

Ornithine, an important precursor for the roasty crust odor, is a non proteinogenic amino acid. It is found in significant amounts in bakers yeast (Schieberle, 1990), and it is generated during microbial metabolism. *L. pontis* is able to metabolize arginine (Vogel et al., 1994) and Ograbek et al. showed 1999 that arginine was converted to ornithine via the arginine deiminase pathway, which involves the three enzymes arginine deiminase, ornithine transcarbamylase, and carbamate kinase. In lactobacilli this pathway was first described for *L. sakei* by Montel and Chapomier (1987).

Numerous procedures especially in France (Baguette), Italy (Ciabatta) and California (San Francisco sourdough French bread) are known to improve flavor and bread texture. These processes have in common, that they use fermentations either of yeasts and/or sourdoughs prior to the dough preparation. These pre-ferments significantly influence the flavor of the bread crumb, the flavor of these breads is richer and more aromatic than in wheat bread (Gassenmeier and Schieberle, 1995; Hansen and Hansen, 1994). In addition to the flavor compounds mentioned earlier, following substances were found in wheat bread crumb prepared from a pre-ferment containing bakers yeasts (FD factor of 64 or higher): methional, ethyl octanoate, 4-vinyl-2-methoxyphenol, and acetic acid (Gassenmeier and Schieberle, 1995). In wheat bread crust of a dough fermented for 240 minutes with bakers yeast, 18 more volatile compounds were detectable, which leads to a more intense roasty odor note (Zehentbauer and Grosch, 1998). Breads prepared from doughs fermented with lactic acid bacteria contained higher amounts of 2/3-methylpropanoic acid, 2/3-methyl-1-butanol, and acetic acid than control breads. A mixture of lactic acid bacteria and yeasts resulted in an increase of 2/3-methyl-butanol, 2-methylpropanoic acid, 3-methylbutanoic acid and 2-phenylethanol (Hansen and Hansen, 1996). These volatile compound have in common that they are produced by microbial metabolism from amino acids. Because of the significant role of amino acids for bread aroma increased amounts of free amino acids in wheat dough may improve the flavor of wheat bread. It was already shown that addition of pure ornithine, leucine, and phenylalanine to yeast dough resulted in an increased

conversion to the flavor volatiles during bread production (Schieberle, 1990; Gassenmeier and Schieberle, 1995).

In addition to metabolic activity an enhanced proteolysis brought about by sourdough fermentation may account for the characteristic sensory properties of sourdough breads compared to breads produced from chemically acidified or yeasted doughs (Spicher et al., 1980; Hansen et al., 1989). The preparation of preferments or the utilization of sourdough fermentation increased proteolysis and amino acid liberation in wheat and rye doughs (Spicher and Nierle, 1983; Gobbetti et al., 1994; Collar et al., 1992). Generally, sourdough fermentations with lactic acid bacteria resulted in an increase of amino acid concentrations during the fermentation time whereas dough fermentation with yeasts reduced the concentration of free amino acids. This enhanced proteolysis during sourdough fermentation may be attributed either to the proteolytic activity of sourdough lactic acid bacteria, or to an enhanced proteolysis by cereal enzymes under the conditions of the sourdough fermentation.

Kratochvil and Holas (1984) characterized the proteolytic activity of rye sour dough during the first 3 hours subject to temperature, pH and used sour dough type. Sterile doughs exhibited the same proteolytic activity as fermentations started with pure lactobacilli. Yeasted doughs were characterized by lower amino acid contents. The proteolytic activity of wheat doughs were previously shown by determination of the increase of trichloroacetic acid - soluble nitrogen or changes in rheological properties were detected (Wang and Grant, 1969, Wu and Hoseney 1989). Recently, proteolytic enzymes associated with wheat gluten have been purified and characterized (Bleukx et al., 1997 Bleukx and Delcour, 2000).

Based on the data available on the proteolytic activity of wheat (sour) doughs it remains unclear whether the proteolysis during sourdough fermentation is attributed to the proteolytic activity of starter cultures or cereal enzymes.

1.4 Proteolytic activity and amino nitrogen demand of lactic acid bacteria

Nitrogen is a growth-limiting factor for lactic acid bacteria in several food ecosystems (Laan et al., 1989). Lactic acid bacteria are known to have multiple amino acid auxotrophies (Chopin, 1993; Mills and Thomas, 1981), up to 18 different amino acids (Detmers et al., 1998) are known as essential for growth and biomass production. Depending on the food ecosystem amino acids are available in different concentration and the degree of polymerization of amino nitrogen is strongly correlated with the enzymatic equipment of the cells. In milk, almost no free amino acids are detectable, all amino nitrogen is found in caseins, the main proteins in milk (Mills and Thomas, 1981). Meat proteins are mainly hydrolyzed by endogenous enzymes, therefore peptides are available for micro-organisms in sufficient amounts (Verplaetse, 1994; Hierro et al., 1999). Unfermented wheat dough already contains overall amino acid concentrations of 400 to 500 mg / kg dough (Collar et al., 1991; Gobbetti et al., 1994), and during dough fermentation this concentration further increases (Kratochvil and Holas, 1984) and enables rapid growth.

Proteolytic breakdown by lactic acid bacteria is by best documented for *Lactococcus lactis* (Kunji et al., 1996; Christensen et al., 1999; Guedon et al., 2001), because this micro-organism and its amino nitrogen metabolism is very important for production and quality of cheese. The structural components of the proteolytic system can be divided into three groups on the basis of their function: (i) proteinases which breakdown caseins to peptides (ii) peptidases which degrade peptides, and (iii) transport systems which translocate the breakdown products across the cytoplasmic membrane. A schematic representation of the proteolytic system is shown in Figure 1.1.

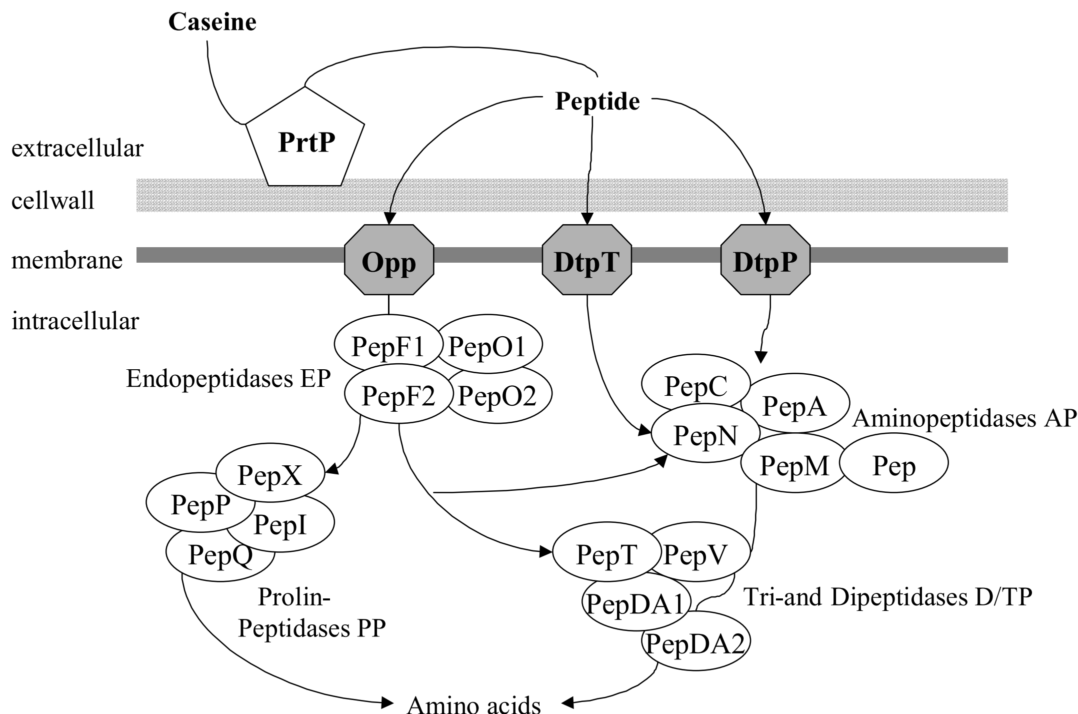

Figure 1.1: Schematic representation of the *Lc. lactis* proteolytic system. The cell wall proteinase, three transport systems and 4 classes of intracellular peptidases are represented in their relative locations in the cell (See Guedon et al., 2001).

The degradation of casein is initiated by an extracellular proteinase PrtP that is bound to the cell wall. Peptides produced by the proteinase are internalized by three transporters, the Opp system takes up oligopeptides of 4 to 18 residues, while DtpT and TtpP transport hydrophilic and hydrophobic di- and tri-peptides, respectively. Intracellular peptides are further hydrolyzed by several intracellular peptidases that are classified depending on their cleavage specificity. Aminopeptidases AP generate dipeptides and amino acids by cleaving the N-terminal end of oligopeptides. Endopeptidases EP cleave internal peptide bonds of oligopeptides, Di/Tri-peptidases D/TP liberates amino acids from di/tri-peptides, and proline peptidases PP are found in hydrolyzing peptides containing proline residues.

Within the lactobacilli genus *L. helveticus*, *L. delbrückii* and *L. casei* are best documented (Gilbert et al., 1996) but additionally informations on the proteolytic degradation of proteins by lactobacilli isolated from meat or sourdough were described. Levels of proteolytic activity in meat and sourdough by lactobacilli were measured by Sanz et al. (1999), Fadda et al., (2001), and Di Cagno et al.(2001)

and only a low hydrolysis of proteins was detected. *L. sanfranciscensis* is the only species isolated from sourdough, where enzymes belonging to the proteolytic system have been purified and characterized (Gobbetti et al., 1996). A cell wall bound proteinase of the serine type was so far described for *L. casei* (Kojic et al., 1991; Naes and Nissen-Meyer, 1992; and Holck and Naes, 1992*),* *L. delbrückii* subsp. *bulgaricus* (Laloi et al., 1991; Gilbert et al., 1996), *L. helveticus* (Zevaco and Gripon, 1988; Yamamoto et al., 1993; Martin-Hernandez et al., 1994), *L. plantarum* (El Soda et al., 1986), and *L. sanfranciscensis* (Gobbetti et al., 1996). About the peptide transport system little is known in lactobacilli, preliminary results and sequence homology suggest, that *L. delbrückii* (Stucky et al., 1995) and *L. helveticus* (Christensen et al., 1995) have at least partially similar transporters. Peptidases were described for *L. casei* (Arora and Lee, 1992), *L. delbrückii* (Klein et al., 1993; Klein et al., 1994; Bockelmann et al., 1992), *L. helveticus* (Christensen et al., 1995; Bosmann et al., 1996), and *L. sanfranciscensis* (Gobbetti et al., 1996).

Current results show, that the expression of the proteolytic system in *Lc. lactis* is strongly regulated by peptides available in the medium. Guedon et al. (2001) have used promoter fusions with luciferase reporter genes to elucidate the regulation of proteases, peptidases and transporter. They have shown, that only the transcription of pepP is modulated by the source of sugar. The expression of prtP (protease), pepC and pepN (both aminopeptidases), pepX (proline peptidase) and the opp-pepO1 operon (oligopeptide transporter and endopeptidase) is repressed 5 to 150 fold when growth on a complex medium is compared to growth on a chemically defined medium. The addition of Prolyl-leucine and leucyl-proline could repress the transcription of latter operons (except of pepX) two-to eightfold. Therefore, at least some peptides internalized by DtpT and DtpP seem to have regulator function on the expression of enzymes and transporters involved in the first breakdown step of proteins.

Proteolytic degradation in sour dough is not only interesting with respect to peptides and amino acids and their influence on flavor. As opposed to rye dough, wheat dough proteins are of particular importance for dough rheology and bread quality and therefore changes in functional properties of gluten proteins catalyzed by proteases are worthwhile to investigate.

1.5 Wheat proteins and gluten macropolymer

Gluten proteins are considered a major determinant for the rheological properties of wheat doughs and the texture of wheat breads attained by straight dough processes. A high correlation is observed between the content of glutenin subunits as well as the glutenin macropolymer (GMP) of flours and their bread making quality (Wieser and Kieffer, 2001; Weegels et al., 1996a). Glutenin subunits aggregate by disulfide bonding to large polymers, the glutenin macropolymer, with molecular masses up to several million (Lindsay and Skerritt, 1999, Weegels et al., 1996a). Depending on the number of cysteine residues available for interprotein disulfide bridges, individual glutenin proteins are thought to act as chain extenders or chain terminators (Lindsay et al., 2000). In addition recent studies indicate, that tyrosine bonds formed in wheat doughs during the processes mixing and baking also contribute to the structure of gluten network (Tilley et al., 2001).

During dough mixing, the GMP partially depolymerizes into aggregates, followed by incorporation of high molecular weight glutenin subunits into the glutenin macropolymer during resting time (Weegels et al., 1997 and 1996b, Lindsay et al., 2000). Gluten strength is furthermore influenced during dough mixing by disulfide interchange of intermolecular glutenin SS bonds with low molecular weight sulfhydril compounds, e.g. cysteine or glutathione (Grosch and Wieser, 1999).

The rheology of wheat doughs in straight dough processes and the resulting loaf volume of bread are mainly determined by gluten proteins. Rheological studies on wheat doughs acidified with lactic and acetic acids, and with sourdoughs demonstrated that the rheological characteristics of doughs changed entirely with fermentation. Major developments included an overall decrease in viscosity which was related to acidification rather than gas production by microorganisms (Wehrle and Arendt, 1998; Wehrle et al., 1997). The loss of extensibility of cracker sponges during fermentation was most pronounced at pH values ranging from 3.8 to 4.1, corresponding to the optimum pH of wheat flour proteinases (Wu and Hoseney, 1989).

To indicate the bread making quality of flours there are some rapid and simple test procedures well established. Flour is suspended in diluted acetic acid or SDS, after centrifugation the glutenin produce a gel-like layer upon the precipitated starch. The amount of this protein layer termed gel protein is in

good correlation with the loaf volume (Sapirstein and Suchy, 1999). The supernatant, which contains all SDS-soluble proteins including albumin, globulin, gliadin and some glutenin polymers can be used for further investigations. The most widely used technique for size distribution and characterization of the polymers is high performance size exclusion chromatography (Dachkevitch and Autran, 1989; Gupta et al, 1993). Carceller and Aussenac (2001) combined this separation method with multi-angle laser light scattering (MALLS) and reported that SDS soluble and insoluble glutenin polymers do not significantly differ in molecular weight, but in molecular structure and density.

1.6 Determination of amino acids in complex media by anion exchange chromatography and integrated pulsed amperometric detection

As described earlier in this work the determination of amino acids is of prime importance. Anion exchange chromatography in combination with integrated pulsed amperometric detection (IPAD) appears to be favorable because this combination offers a very sensitive method to separate and detect amino acids (Clarke et al., 1999). This method is also used for the detection of carbohydrates and amines (Johnson et al., 1993). The simultaneous analysis of these analytes is advantageous for samples with approximately the same concentration levels of amino acids, sugars, and sugar alcohols, for example samples from cell cultures or bacterial fermentations (Hanko and Rohrer, 2000).

Food samples such as fruit juices or cereal extracts typically contain much higher concentrations of sugars compared to the free amino acid content. In anion exchange chromatography separations combined with IPAD glucose, fructose, and sucrose interfere with the determination of amino acids because they co elute with asparagine, alanine, threonine, and serine (Eggleston, 1999). Not only the accurate quantitative and specific determination of amino acids is unattainable but also the merely qualitative estimation of their contents is impossible. Hence a sample preparation is required prior to HPLC analysis.

Amino acids, sugars and sugar alcohols may be separated on ion exchange solid phase extraction systems based on their different polar and ionic properties. Amino acids are amphoteric ions, and dependent on the pH value of the solution they act as cations or anions. Over a wide range of

pH-values sugars are uncharged and do not interact with ionic matrices. Thus, appropriate selection of ion exchange resins will enable to separate carbohydrates and amino acids for subsequent analysis by anion exchange chromatography / IPAD detection.

Recently, a method for quantification of amino acids from samples with high monosaccharide contents was described by Jandik et al. (2001). They captured the amino acids on a cation-exchange HPLC column under conditions where carbohydrates do not bind to the column. After elution of carbohydrates, the eluent flow direction changes and amino acids are applied to the anion exchange column.

Because arginine is co eluted with the sample matrix in the void volume, matrix components interfere with an accurate determination unless they are completely removed by an appropriate sample preparation procedure. A new technique for increasing the retention of arginine was already described by Jandik et al. (2000). However, this technique eliminates carbohydrates from the sample and allows interference-free analysis of only amino acids. Furthermore, the method has instrumental requirements in addition to the hard and software for anion exchange amino acid separations and therefore does not offer the flexibility required for applications in research laboratories or occasional samples in quality control.

1.7 Aim of this work

The aim of this work was to elucidate the influence of sourdough flora and wheat enzymes on flavor of bread and on rheology of wheat dough. Based on the information, that amino acids are the precursors of the flavor active compounds, especially of the roasty crust note, work should be done on flavor improvement, dependent on fermentation conditions and strains. Naturally, a main focus should be on the amino nitrogen demand and uptake of lactic acid bacteria and on the differences between lactic acid bacteria and yeast. A screening for strains with an improved proteolytic activity should be performed and should allow to increase the amount of precursors.

To enable these investigations, sample preparation for the fast and flexible separation of sugars and amino acids, including arginine, had to be established.

Another emphasis of this work was to determine the influence of sourdough fermentation and lactic acid bacteria on gluten proteins. Modifications on single proteins as well as changes on the gluten network were expected. These influences on the proteins should determined with classical methods and a method using FITC-labeled protein.

The overall aim of this work was to obtain insight in the respective role of micro-organisms, endogenous enzymes and fermentation conditions on changes of wheat proteins, peptides and amino acids during sourdough fermentation.

2. Materials and Methods

2.1 General Methods

2.1.1 Strains and culture conditions.

The microorganisms used in this study for sourdough fermentations are shown in Table 2.1.

Table 2.1. Microorganisms and culture conditions

Organism	Strain	Reference	culture conditions
Lactobacillus pontis	DSM 8475^T TMW 1.397	Vogel et al. (1994)	Homiochii or mMRS4 anaerobic 37 °C
Lactobacillus sanfranciscensis	ATTC 27651 TMW 1.53		Homiochii or mMRS4 anaerobic 30 °C
L. sanfranciscensis	LTH2581 TMW 1.52	Böcker et al. (1990)	Homiochii or mMRS4 anaerobic 30 °C
Candida milleri	TMW 3.139		mMRS4, aerobic 20°C
Saccharomyces cerevisiae	NCYC 1200		mMRS4, aerobic 20°C

All strains were sourdough isolates. *Lactobacillus pontis* TMW 1.397 was selected because of its ability to convert arginine to ornithine (Vogel et al., 1994). *Lactobacillus sanfranciscensis* LTH2581, previously designated *Lactobacillus brevis var. lindneri* II, is a constituent of a commercially available sourdough starter (Böcker et al., 1990). Lactobacilli were cultivated either on Homiochii medium or on mMRS4 (Stolz et al., 1993) and plates were incubated under modified atmosphere (4% O_2, 20% CO_2, and 76% N_2) whereas *L. sanfranciscensis* was grown at 30 °C and *L. pontis* at 37 °C. The yeasts *Candida milleri* TMW 3.139 and *Saccharomyces cerevisiae* NCYC 1200 were grown on modified mMRS4 at room temperature.

2.1.2 Media

Homiochii medium Homiochii medium contained the following per liter: 7.0 g glucose x H_2O, 7.0 g fructose, 7.0 g maltose, 10.0 g peptone from casein, 2.0 g meat extract, 7.0 g yeast extract, 5.0 g sodium-acetate trihydrate, 5.0 g $(NH_3)_2$ - citrate, 5.0 g KH_2PO_4, 2.0 g Na-gluconate, 0.5 g L-cysteine

HClxH_2O, 0.2 g $MgSO_4$ x 7 H_2O, 0.1 g $MnSO_4$ x H_2O, 0.05 g $FeSO_4$ x 7 H_2O, 1.0 g Tween 80. The pH was adjusted to 5.4. Solid media additionally contained 17.0 g l^{-1} agar.

mMRS4 medium Modified MRS4 medium contained the following per liter: 5.0 g glucose x H_2O, 10.0 g fructose, 10.0 g maltose, 10.0 g peptone from casein, 5.0 g meat extract, 5.0 g yeast extract, 4.0 g KH_2PO_4, 2.6 g K_2HPO_4 x 3 H_2O, 3,0 g NH_4Cl, 1 ml Tween 80, 0.1 g $MgSO_4$ x 7 H_2O, 0.05 g $MnSO_4$ x H_2O, 0.5 g L-cysteine HCl x H_2O, 0,2 mg each of biotin, folic acid, nicotinic acid, pyridoxal phosphate, thiamin, riboflavin, cobalamin and panthothenic acid. The pH was adjusted to 6.0, solid media additionally contained 17.0 g L^{-1} agar.

2.1.3 Determination of viable cell counts, and pH.

Cell counts were determined by plating appropriate dilutions of dough on MRS4 agar and Homiochii agar. The media contained 20 mg/l chloramphenicol or 75 mg/l cycloheximide for selective enumeration of yeasts and lactic acid bacteria, respectively. To determine the dough pH, 1.0 g of dough was mixed with 9 mL of demineralized water, vortexed for 5 min, and the pH was measured with a glass electrode.

2.1.4 Determination of carbohydrates and organic acids in dough.

To determine the concentration of organic acids, alcohols and carbohydrates, 500 mg dough samples were thoroughly mixed with 500 µl 7% perchloric acid and stored overnight at 4 °C. The precipitate was removed by centrifugation at 15000 x g for 10 min and the clear supernatant was used for analysis by HPLC. 20 µl Samples were injected on a Polyspher^R OAKC column (300 x 7.8 mm, Merck, Darmstadt) connected to a refractive index detector and eluted with 5 mM H_2SO_4 at a flow of 0.4 ml min^{-1}. The column temperature was maintained at 70 °C.

2.1.5 Determination of total amino nitrogen content

A modified ninhydrin method was used for determination of total amino nitrogen (Drawert, 1987; Thiele et al., 2002). Dough samples were clarified with perchloric acid as described above. Clear supernatant (100 µl) was mixed with 20 µl 3 M KCl to precipitate the perchloric acid. After 1 h at room temperature, the precipitate was removed by centrifugation (10 min at 15000 x g). Reagent 1 (100 µl, 5.0 g Na_2HPO_4 *2 H_2O, 6.0 g KH_2PO_4, 0.5 g ninhydrin and 0.3 g fructose in 100 ml $H_2O_{bidest.}$,

pH 6.7) was mixed with 10 µl sample and 190 µl H_2O_{bidest}, heated for 16 minutes at 100 °C. Reagent 2 (500 µl, 0.2 g KIO_3 solved in 60 ml bidistilled water and 40 ml 98% ethanol) were added, the sample mixed thoroughly, and the adsorbance of the solution was measured at 570 nm. A calibration curve was prepared with each measurement using glycine as standard and results were expressed as mmol glycine l^{-1}. The coefficient of variation of the assay was generally less than 5 % and sampled from duplicate sourdough fermentation were reproducible with a coefficient of variation of 10 % or less.

2.1.6 Size exclusion chromatography (SEC) of SDS-soluble proteins and SDS-DTT soluble proteins.

Dough samples were characterized by SEC with respect to the amount of SDS-soluble proteins as well as their size distribution. One g of dough was extracted with 4 mL of 1.5% sodium dodecyl sulfate (SDS) in 50 mM sodium phosphate buffer, pH 6.9 (Lindsay et al., 2000). Proteins in the residue of this first extraction were subsequently solubilized in 1.5% SDS in 50 mM phosphate buffer, pH 6.9, additionally containing 4% dithiothreitol (DTT). SDS-extracts were applied on a Superdex 200 column coupled to a Superdex Peptide column (both from Amersham biosciences, Uppsala, Sweden) to achieve fractionation in the molecular weight (MW) range of 100 to 5 x 10^6. SDS-DTT extracts were applied to a Superdex 200 column to achieve fractionation in the MW range of 10^4 to 5 x 10^6 Da. Samples were eluted with 50 mM sodium phosphate buffer, pH 7.7, containing 0.1% SDS and 20% acetonitrile at a flow of 0.4 mL / min. The UV detector was set to 210 and 280 nm. The areas under the peaks representing the polymeric, monomeric, and low molecular weight (LMW) components were determined using the 280 nm trace. The column(s) were calibrated using the HMS and LMW GPC calibration kits (Amersham biosciences, Uppsala, Sweden). Additionally, the elution volumes of polymeric and monomeric proteins, and LMW wheat proteins were determined by analysis of SDS-extracts from unfermented flour. Fractions were collected and analyzed by native SDS-PAGE as described below. Polymeric proteins eluted at 12 mL (void volume) to 18 mL, monomeric proteins eluted at 18 to 30 mL, amino acids and peptides with a molecular weight of less than 5000 eluted at 30 to 44 mL (total column volume). The experimental error of dough extraction, chromatographic

separaration, and peak integration was less than 10%, and to independent fermentations yielded qualitative and quantitative consistent results.

2.1.7 SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE analysis of flour proteins was performed in a Bio-Rad Mini-Protean vertical electrophoresis cell (Bio-Rad, Munich, Germany) using the Laemmli buffer system according to the instructions of the manufacturer. Dough extracts were mixed with 3 vol of sample buffer and heated to 90°C for 5 min in the presence of 5% mercaptoethanol, separated in 12% acrylamide, 2.67% cross-linked gels, and stained with Coomassie-Blue. Heating of samples in the presence of mercaptoethanol was omitted for native SDS-PAGE of gluten polymers. Gels containing fluorescence labeled proteins were additionally visualized by UV transillumination and documented with a video imaging system (Herolab, Wiesloch, Germany). All gels were stained with Coomassie-Blue. Heating of samples in the presence of mercaptoethanol was omitted for native SDS-PAGE of gluten polymers. Independent fermentations yielded qualitative consistent results.

2.2 Generation of flavor precursor compounds during sourdough fermentation

2.2.1 Sourdough fermentation.

The wheat flour used was obtained at a local mill and contained 13.6% moisture, 34% wet gluten and 0.98% ash. Starter cultures were grown overnight, harvested at 15000 x g for 10 minutes, washed once with sterile tap water and then resuspended in the same volume sterile tap water. Doughs with a dough yield of 400 were prepared according to the formulas shown in Table 2.2 in 50 ml glass beakers, stirred to homogeneity with a spatula, covered and incubated at 30°C.

Table 2.2. Dough formulas for preparation of sourdoughs.

	sterile	**sterile, salt**	**sterile, acid**	**sterile, DTT**	**yeast**	**mixed culture**	**LAB**
wheat flour	10 g	10 g	10 g	10 g	10 g	10 g	10 g
tap water	30 g	30 g	30 g	30 g	26 g	22 g	26 g
Chloramphenicol (100 mg/kg dough)	4 mg	4 mg	4 mg	4 mg	4 mg		
Erythromycin (100 mg/kg dough)	4 mg	4 mg	4 mg	4 mg			
Cycloheximide (150 mg/kg dough)	6 mg	6 mg	6 mg	6 mg			
NaCl (10 g/kg dough)		0.4 g					
lactic and acetic acids [a)]			180 µl				
DTT (1.9 g/kg dough)				77 mg			
C. milleri or *S. cerevisiae* (10^9 CFU/kg dough)					4 ml	4 ml	
L. sanfranciscensis or *L. pontis* (10^{11} CFU/kg dough)						4 ml	4 ml

[a)] mixture of 4 vol. lactic acid (90%) and 1 vol acetic acid (98%).

Samples were taken of appropriate intervals to determine viable cell counts, pH, as well as the concentrations of organic acids, ethanol, and amino acids. The absence of contaminants in sourdough fermentation was verified by determination of the colony morphology and microscopy of selected colonies for the determination of the cell morphology. Each fermentation was carried out in duplicate or quadruplicate as indicated in the figure legends.

2.2.2 Baking experiments.

Preferments were prepared with 480 g wheat flour, 120 ml cell suspension and 600 g tap water, mixed for 2 minutes at medium speed and 1 minute at high speed with a Hobart kneader and incubated at 30 °C, and 80% relative humidity. Bread dough was prepared according to the following formula: 3000 g flour, 1740 g tap water, 150 g pressed yeast, 60 g salt, 16.2 g glucose, and 27 g of a commercially available baking aid devoid of protease activities (composition according to the supplier: 30% soy lecithin, 30% guar flour, 30% gelatinized flour, 8% vegetable fat, 1% ascorbic acid, 0.25% α-amylase

and 1% of a hemicellulase preparation). Preferments were prepared as indicated in Table 2.3 and the dough formulas were corrected for the amounts of flour and water added with the preferment.

Table 2.3. Preparation of preferments for baking experiments.

	Starter culture	**fermentation time**	**% preferment addition** [a)]	**Additives**
D1	none (control)	-	-	-
D2	none (control, low amino acids)	-	-	Amino acids [b)]
D3	none (control, high amino acids)	-	-	Amino acids [c)]
D4	none (chemically acidified)	40 h	13 %	lactic and acetic acids
D5	*S. cerevisiae*	24 h	13 %	
D6	*C. milleri*	24 h	13 %	
D7	*L. sanfranciscensis*	40 h	13 %	
D8	*L. sanfranciscensis* + ornithine	40 h	13 %	Ornithine
D9	*L. pontis*	40 h	13 %	
D10	*L. pontis*	20 h	7 %	
D11	*L. pontis*	40 h	7 %	
D12	*L. pontis*	40 h	3.5 %	
D13	*L. pontis*	40 h	13 %	

a) Percent of the flour used for preparation of preferment

b) 0.23 mmol ornithine, 0.53 mmol leucine, 0.30 mmol phenylalanine, 0.23 mmol isoleucine, and 0.13 mmol methionine per kg flour.

c) 10 mmol of these amino acids per kg flour

Bread doughs were kneaded with a Diosna spiral kneader, 2 minutes at low speed and 5 minutes at high speed. First proofing of the doughs lasted 20 minutes at 25 °C, second proofing of the formed rolls took 45 minutes at 30°C and 80% relative humidity. The rolls were baked for 17 minutes at 240 °C at three different places in the oven to rule out possible effects of heat and moisture variations within the oven. After 1 hour cooling at room temperature, sensory evaluation was performed by an expert panel consisting of 3 to 8 persons. Three batches of dough and the control dough (straight dough process without preferment or additives) were prepared on a single day. The panelists were

asked to score the aroma intensity of these four samples and to describe the crust odor in comparison to that of the control rolls.

2.2.3 Amino acid analysis by post column derivatization

To determine the amino acid concentration, dough (500 mg) were mixed with 500 µl of 96% ethanol. The sample was incubated for one hour at 4°C to prevent proteolysis during sample extraction. After centrifugation 500 µl of supernatant was freeze dried. It was verified by extraction of dough samples and cultures of *L. sanfranciscensis* and *Saccharomyces cerevisiae* in mMRS4 that this extraction procedure provided the same amino acid recovery from doughs as the extraction procedure previously used by Collar et al. (1991) and additionally recovered those amino acids accumulated intracellularly by the fermentative microorganisms (data not shown). The sample were analyzed on a cation exchange column by an automated amino acid analyzer LC3000 (Eppendorf Biotronik, Germany) using ninhydrin post column derivatization essentially according to Spackmann et al. (1958). 20 µl sample were injected on a BTC.2410 column (125x4.6 mm, 4µm particle size, 10% cross-linked) and eluted at 0.25 ml min^{-1} with the following solvents and column temperatures: buffer A (8.2 g l^{-1} Na-acetate, 75 ml l^{-1} methanol, 3 ml l^{-1} formic acid, 15 ml l^{-1} acetic acid, 100 µl l^{-1} octanoic acid, pH 3.3) 10 min, 47°C; buffer B (8.2 g l^{-1} Na-acetate, 3 ml l^{-1} formic acid, 20 ml l^{-1} acetic acid, 100 µl l^{-1} octanoic acid, pH 3.6) 6 min, 48°C; buffer C (8.2 g l^{-1} Na-acetate, 2 ml l^{-1} formic acid, 1.5 ml l^{-1} acetic acid, 100 µl l^{-1} octanoic acid, pH 4.5) 9 min, 49°C; buffer C, 14 min, 50°C min; buffer D (8.2 g l^{-1} Na-acetate, 1.2 ml l^{-1} formic acid, 5 ml l^{-1} acetic acid, 2 g l^{-1} boric acid, Na_2EDTA 0.5 g l^{-1}, NaOH 6 g l^{-1}, 100 µl l^{-1} octanoic acid, pH 11.0) 15 min, 52°C; buffer E (as buffer D, pH 11.6) 10 min, 56°C, buffer E: 18 min, 60°C. Derivatization with ninhydrin was achieved with a flow of 0.25 ml min^{-1} of ninhydrin reagent (20 g ninhydrin, 0.6 g hydrindantin dihydrate, 50 ml tetrahydrofuran, 500 ml ethyleneglycol, 450 ml 5.1 molar K-acetate, pH 5.59) at 125 °C. Proline was detected by the UV-absorption at 440 nm and all other amino acids at 570 nm. The coefficient of variation of the analysis was generally less than 2% and samples from duplicate sourdough fermentations were reproducible with a coefficient of variation of 6 % or less.

2.3 Screening for proteolytic active microorganisms

2.3.1 Strains and culture conditions

To select strains of lactic acid bacteria, which have a increased proteolytic activity, 82 different lactic acid bacteria were chosen from the TMW strain collection. A strain of each available lactic acid bacteria species and a variety of lactobacilli strains isolated from sourdough were selected. Following species were used: *L. acidophilus, L. alimentarius, L. amylolyticus, L. amylovorus, L. animalis, L. brevis, L. buchneri, L. casei, L. cerealis, L. curvatus, L. delbrückii ssp bulgaricus, L. delbrückii ssp. delbrueckii, L. delbrückii ssp. lactis, L. farciminis, L. fermentum, L. fructivorans, L. fructosus, L. frumentii, L. helveticus, L. kandleri, L. manihotivorans, , L. oris, L. panis, L. paracasei ssp. Paracasei, L. pentosus, L. plantarum, L. pontis, L. reuteri, L. rhamnosus, L. sakei, L. sanfranciscensis, L. viridens, Leuconostoc mesenteroides, Pediococcus pentosaceus, Weissella confusa, and Enterococcus faecium*. A detailed list including strain numbers is given in table 3.2 in the result section. Strains were cultivated on Homiochii broth in 10 ml screw cap glass tubes until all cultures reached stationary phase. Cultures were centrifuged, resuspended in 0.5 ml fresh Homiochii broth and 40 % glycerol, each 200 µl were transferred in duplicate to microtiter plates and stored at – 80 °C. This plates were called master plates.

2.3.2 Media

Gluten-agar. Gluten-agar was prepared according to Wiese (1995) with some modifications concerning maltose, fructose, and yeast extract to enable growth for lactic acid bacteria and contained the following per liter: 15,0 g gluten (Sigma), 10.0 g glucose x H_2O, 5.0 g fructose, 5.0 g maltose, 3.0 g yeast extract, 2.0 g K_2HPO_4 x 3 H_2O, 1 g Tween 80, 0.1 g $MgSO_4$ x 7 H_2O, 0.05 g $MnSO_4$ x H_2O, 0.5 g L-cysteine HCl x H_2O, 17.0 g agar. The pH was adjusted to 6.0 and media was well resuspended prior casting to achieve constant cloudy plates.

Calcium-caseinate-agar. Calcium-caseinate agar was prepared according to Frazier and Rupp (1993) with some modifications concerning all sugars, yeast extract, milk powder, vitamins and trace elements. The medium contained the following per liter: 5.9 g peptone, 3.0 g meat extract, 5.0 g

sodium chloride, 2.5 g peptone from casein according to Hammarsten, 0.15 g calcium hydroxide, 0.05 g calcium chloride, 3.0 g yeast extract, 5.0 g skimmed milk powder, 2.0 g glucose x H_2O, 2.0 g maltose, 2.0 g fructose, 0.1 g $MgSO_4$ x 7 H_2O, 0.05 g $MnSO_4$ x H_2O, 0,2 mg each of biotin, folic acid, nicotinic acid, pyridoxal phosphate, thiamin, riboflavin, cobalamin and panthothenic acid, 17.0 g agar. All ingredients were solved in 1.0 L demineralized water, well suspended and the pH was adjusted to 6.0. After 30 minutes resting time, medium was permanently stirred, slowly heated and boiled for 15 minutes. Coarse particles were removed by filtering through gauze. After sterilization the media should be cloudy.

2.3.3 Screening on agar plates

All strains from the master plates were screened on two types of agar plates, gluten agar and calcium-caseinate agar. Both media contained suspended solid particle which made the agar plates cloudy. Proteolytic activity results in clear zones around the colonies. As control for growth and the absence of contaminants microorganisms were also cultured on Homiochii plates. Inoculation of agar plates were performed directly from the master plates. The microtiter plates were stored on ice to prevent thawing. The culture suspension was transferred to Homiochii agar, gluten agar and calcium-caseinate agar in one step, whereas the pipette tips were moved over the surface. After 2 days plates were controlled in respect to growth, contaminants and clearance of the media. As positive control the *Bacillus subtilis* type strain was used.

2.3.4 Determination of proteolytic activity by fluorescent substrates

Preparation of FITC-casein

FITC-casein was prepared according to Twining (1984). One gram of casein was dissolved in 100 ml of 50 mM carbonate buffer, pH 9.5, containing 150 mM NaCl. This solution was warmed to solve the proteins and afterwards cooled to room temperature. To this solution 40 mg FITC was added and the mixture was gently stirred for 1 h at room temperature. Free FITC was removed by dialyzing twice against suspensions of charcoal in 2 liters water at 4 °C, then against 50 mM Tris buffer pH 8.5, followed by 50 mM Tris buffer pH 7.2. The protein concentration was adjusted to 0.5% with 50 mM Tris buffer, pH 7.2. The substrate was stored in aliquots at – 20 °C.

Determination of proteolytic activity

Proteolytic activity was determined for L. sanfranciscensis TMW 1.52 and 1.53. As positive control *B. subtilis* and two proteases, a fungal protease and papain were used.

50 µl of a *B. subtilis* culture was incubated for 200 minutes in 50 µl water and 100 µl FITC-casein and the fluorescence was detected on-line in a TECAN spectrafluor at emission/ excitation wavelength of 485/520, Gain 40 and 3 flashes.

All other fermentations were performed with a buffer system, containing 50 mM KH_2PO_4 100 mM Na_3 citrate, 0.1 g L^{-1} $MgSO_4$ x 7 H_2O, 0.05 g $L^{-1}$$MnSO_4$ x H_2O, pH was adjusted to 7.0, 6.0, and 5.0. Sugar solution contained 35 mM maltose and 70 mM fructose. Microorganism were fermented for 6 hours in 10 ml Homiochii medium, after centrifugation they were resuspended in 1 ml 0.9% sodium chloride and immediately used for inoculation of microtiter plates. Fermentations were performed at 30 °C in a TECAN spectrafluor and formulas for fermentations are shown in Table 2.4.

Table 2.4. Formulas for fermentations determining the proteolytic activity of *L. sanfranciscensis* strains

	Aseptic	Aseptic + protease	Fermented with 1.52 or 1.53	Fermented with 1.52 or 1.53 + fungal protease
Buffer (µl)	100	100	100	100
Microorganisms (µl)	0	0	10	10
Protease (µl)	0	10	0	10
sugar solution (µl)	2	2	2	2
FITC-proteins (µl)	75	75	75	75
demineralized water (µl)	20	10	10	10

2.4 Nitrogen demand and peptide utilization by L. sanfranciscensis

2.4.1 Amino nitrogen free media

Amino nitrogen free media was prepared according to mMRS4 without amino nitrogen and with a increased buffer capacity. The media contained the following per liter: 5.0 g glucose x H_2O, 10.0 g fructose, 10.0 g maltose, 4.0 g KH_2PO_4, 2.6 g K_2HPO_4 x 3 H_2O, 6.8 g Na-acetate x 3 H_2O, 3.0 g NH_4Cl, 1 ml Tween 80, 0.2 g $MgSO_4$ x 7 H_2O, 0.05 g $MnSO_4$ x H_2O, 0.05 g $FeSO_4$ x 4 H_2O, 0.5 g L-

cysteine HCl x H_2O, 0,2 mg each of biotin, folic acid, nicotinic acid, pyridoxal phosphate, thiamin, riboflavin, cobalamin and panthothenic acid. The pH was adjusted with acetic acid to 5.5.

2.4.2 Amino nitrogen substrate

To supply micro-organisms with a basic amount of amino acids, yeast extract was enzymatically hydrolyzed. 10 % w/v yeast extract in demineralized water was adjusted to pH 7.0, 5 mg proteinase K and 10 mg Flavourzyme (Protease with Peptidase activity) were added and incubated for 400 minutes at 40 °C. For a complete enzyme inactivation yeast extract was sterilized for 10 minutes at 100°C.

2.4.3 Doughs for peptide extraction

Three different doughs containing *L. pontis* (P), *S. cerevisiae* (H) and a aseptic acidified dough (S) at pH 3.9 were fermented for peptide and amino acid extraction. The used wheat flour 1050 was bought in a local supermarket, dough was prepared with a dough yield of 200, doughs P and S were fermented at 37 °C and dough H at room temperature for 48 hours. After 48 hours the doughs were characterized with respect to pH, cell counts and morphology of colonies on a mMRS4 agar plates.

100 g dough was extracted with 400 ml Triethylamine buffer(pH 7.0 adjusted with CO2 and 25% i-propanol). Dough-extracts were stored over night at 4°C to precipitate polysaccharides and afterwards centrifuged for 15 min. Supernatant was dried in a rotavapor at 55 °C and extracts were completely solved in the smallest possible amount of water. Dough-extracts were aliquoted and stored frozen.

2.4.4 Preparative size exclusion chromatography

Dough-extracts were fractionated using preparative size exclusion chromatography consisting of Sephadex G50 in a column 100 cm x 1.6 cm (all Amersham biosciences) and a solvent which was identical with the extraction buffer. 3 ml of dough extracts were injected and eluted with a flow of 0.5 ml/min. Samples were collected in fractions, freeze-dried and resolved in demineralized water. Fractions were characterized with respect to size distribution and amount using the SEC method described in chapter 2.1.

2.4.5 Cation exchange chromatography

Ion exchange chromatography (CIEX) was performed using a cation exchange column (Bio-Rad Econo-Pac Cartridges High S) and two buffers, buffer A consisting 0.1 M o-phosphoric acid (pH 1.5) and 25% i-propanol and buffer B consisting of 4 M ammonia (pH 12.4). The columns were rinsed with 20 ml demineralized water, dough extracts were diluted 1:1 with buffer A and 3 ml were applied to the columns. Column was first eluted with 10 ml buffer A, the eluent was collected (sample A), than 5 ml buffer B were applied 2 times and eluent was again collected (B1 and B2). All samples were dried in a rotavapor, resuspended in water and afterwards freeze-dried to remove propanol and ammonia. Samples were characterized with respect to size distribution and amount using the SEC method described in chapter 2.1.

2.4.6 Reverse-phase chromatography

After IEX sample A was applied to reverse-phase chromatography (RPC) using a C18-column (Phenomenex, Jupiter 10 µm 300 A 250x4,6) and two eluents, eluent A was 0.1% trifluoroacetic acid (TFA) in demineralized water and eluent B was 0.1 % TFA and 80 % acetonitrile. The gradient is shown in Table 2.5.

Table 2.5: Gradient used for RPC of dough samples after SEC

Elution time (min)	% eluent A	% eluent B
-5	100	0
2	90	10
10	0	100
35	0	100
36	100	0

200 ml of sample A was diluted with 250 ml eluent A and 250 µl were injected. Samples were again fractionated and fractions belonging to the same peak were pooled and dried in a rotavapor. Samples were resolved in demineralized water and characterized by SEC.

2.4.7 Growth experiments with dough fractions

Growth experiments were performed with *L. sanfranciscensis* in microtiter plates at 30 °C and growth was determined using the adsorbance of cells at 590 nm. For each set of experiments a positive as well as a negative control were performed, these results were used for the calculation of relative growth. All fractionated dough samples were adjusted to the same nitrogen content, whereas the nitrogen content was determined by SEC and adsorption measurement at 210 nm.

Prior to growth experiments, *L. sanfranciscensis* was cultivated for 16 hours in mMRS4 to achieve cells within the stationary growth phase. Culture broth was used without washing step and all media were pre-incubated at 30 °C to minimize lag-phase. Growth broth consisted of 150 µl amino nitrogen free media, 20 µl of dough extract, 10 µl demineralized water and 20 µl L. sanfranciscensis preculture.

2.5 De-/polymerization and proteolysis of gluten proteins in sourdough

2.5.1 Sourdough fermentation

A commercial wheat flour with an ash content of 0.55% and protein content of 12.3% was obtained at a local mill. To obtain inocula for sourdoughs, about 10^9 cells from an overnight culture were washed in sterile tap water, resuspended in 2 ml sterile tap water, and used immediately to inoculate the doughs. Doughs were prepared according to the dough formulas shown in Table 2.6, mixed to homogeneity with a spatula (3 min mixing time), and incubated in glass beakers at 30°C. Samples were taken at 0, 6, and 24h for the subsequent analyses. Two independent fermentations were carried out.

Table 2.6. Dough formulas for preparation of control doughs and sourdoughs

	control neutral	control acid	LAB [a)]	LAB, buffer
flour [g]	20	20	20	20
NaCl [g]	0.4	0.4	0.4	0.4
Water [ml]	20	20	18	16
erythromycin [mg]	2	-	-	-
lactic and acetic acids [mg][b)]	0.360	-	-	-
preculture [ml] (10^8 CFU/g dough)	-	-	2	2
phosphate buffer [ml][c)]	-	-	-	2

[a)] *L. sanfranciscensis* LTH2581, or *L. pontis* LTH2587. Cells were harvested from 5 ml preculture and washed twice with tap water

[b)] Mixture of 4 volumes of lactic acid (90%) and 1 volume of acetic acid (98%).

[c)] 1 M Sodium phosphate, pH adjusted to 6.3 with NaOH.

2.5.2 Extraction of doughs and reversed-phase (RP)-HPLC analysis of dough extracts.

Sequential extraction of dough samples was performed essentially according to Kruger et al. (1988) with the solvents 1) 0.5 M NaCl, 150 mM sodium phosphate, pH 6.8; 2) 50% 1-propanol in H_2O_{bidest}, and 3) 50% 1-propanol in H_2O_{bidest}, 1% acetic acid and 4% dithiothreitol (DTT). To 2 g of dough were added 6 mL of solvent, the suspension was thoroughly mixed and incubated for 30 min at ambient temperature in an overhead mixer. It was verified that the buffering capacity of solvent 1 sufficed to compensate differences in pH of the various neutral and acidic dough samples. A 4 mL water wash was included between solvent 1 and solvent 2 to remove salt and residual organic acids. Dough extracts were stored at –20°C in the dark until analysis by SDS-PAGE or HPLC.

For quantitative analysis of the protein content of propanol-water and propanol - acetic acid – DTT extracts, reversed-phase(RP) HPLC was performed according to Wieser et al. (1998). 20 µL samples were injected on a C18 RP column (250 x 4.6 mm, 300 Å, 10 µm particle size, Phenomenex, Torrence, U.S.A.) coupled to a UV detector set to 210 nm, the flow was 1 mL / min, and the column temperature was 70°C. Solvents A and B consisted of 0.1% trifluoroacetic acid in water and acetonitrile, respectively. Samples were eluted with the following solvent gradient: 0 to 2 min: 24% B,

2 – 32 min: gradient from 24% to 56% B. The experimental error of dough extraction, chromatographic separation, and integration was less than 5%, and to independent fermentations yielded qualitative and quantitative consistent results.

2.6 De-/Polymerization and proteolysis determined with FITC labeled wheat proteins

2.6.1 Extraction of wheat proteins.

A commercially available wheat flour with an ash content of 1.05 % was bought in a supermarket. Sequential extraction of wheat proteins was performed according to Kruger et al. (1988) with one modification: glutenin was extracted with two different solvents, one containing dithiothreitol (DTT) another one without DTT but with sodium dodecyl sulfate (SDS). Following solvents were used: 1) 0.5 M NaCl, 150 mM sodium phosphate, pH 6.8; 2) 50% n-propanol in H_2O_{bidest}, 3A) 50% n-propanol in H_2O_{bidest}, 1% acetic acid and 4% DTT, and 3B) 1.5% SDS in 50 mM sodium phosphate buffer, pH 6.9. To 10 g of wheat were added 40 ml of solvent, the suspension was thoroughly mixed and incubated for 30 min at 4°C in an overhead mixer. Low temperature was used to reduce proteolytic degradation of the proteins. Extraction with each solvent was performed twice. Between solvent 1 and solvent 2 a 40 ml water wash was included to remove all salt from solvent 1. After solvent 2, the pellet was divided into equal portions and extracted either with 20 ml solvent 3A or solvent 3B. All extracts were stored at –20°C .

2.6.2 Labeling of wheat proteins with FITC.

Labeling with FITC was performed essentially according to Lindsay et al. (2000). 20 ml fractions were dried in a rotavapor and the residues were solved in 6 ml 100mM carbonate buffer (albumin/globulin, pH 9.0) or 50% propanol, 100 mM carbonate buffer, pH 9.0 (gliadin and glutenin). 3 mL 1% w/v FITC in dimethylsulfoxid was added to each sample under stirring and labeling was performed for 2 hours at room temperature.

2.6.3 Purification of labeled proteins.

After labeling samples were dialyzed at 4 °C 24 hours against the extraction buffer. Buffers were changed 4 times and the first two cleaning steps contained activated charcoal. For a complete removal of FITC all samples were additionally separated by gel permeation chromatography using Sephadex

G-10 (column 7 mm X 200 mm, all Amersham biosciences, Uppsala, Sweden) and eluted with 20% acetonitrile in 50 mM triethylamine buffer, pH was adjusted to about 7.0 using CO_2. 2.5 mL sample were diluted with 1.5 mL solvent, applied to the column and eluent was fractionated. Fractions containing proteins but no free FITC were pooled, lyophilized, and stored frozen. All labeling and purification steps were performed without precipitation of the protein solution.

2.6.4 Reconstitution of wheat doughs with fluorescent labeled proteins and dough fermentation

Fermentations were performed using sourdough isolates *Lactobacillus sanfranciscensis* LTH2581 (Böcker et al., 1990) and *Lactobacillus pontis* TMW 1.397 (=DSM 8475^T, Vogel et al., 1994), grown in modified MRS. Inocula for sourdough experiments were prepared by two sequential incubations for 24 h. Cells from 1 ml culture (about 10^9 cells) were washed in sterile tab water, resuspended in 1 ml tab water, and used immediately to inoculate the doughs. Doughs with a dough yield of 225 were prepared according to the dough formulas shown in Table 2.7, mixed to homogeneity with a spatula (1 min mixing time) and incubated in Eppendorf tubes at 30 °C. Samples of about 200 mg were taken at 0, 7, and 24 hours and extracted immediately.

The addition of labeled protein fractions to different dough systems was performed in 6 sets of experiments with 4 different protein additions. The following protein additions were used: FITC-albumin/globulin, FITC-gliadin, FITC-glutenin, and addition of water served as control. The sets of experiments differed in the fermentation conditions: control fermentations with or without acidification where microbial growth and metabolism was inhibited (less than 10^4 colony forming units per g dough), sourdough fermentations with *L. pontis* or *L. sanfranciscensis*, and addition of protease to an acid control dough and a dough fermented with *L. pontis*. Samples were taken from the doughs after 0, 7, and 24 hours of fermentation. Cell counts were determined as described (Thiele et al., 2002). Dough samples were extracted immediately with 1.5 % SDS fur subsequent SEC analysis as described below.

Table 2.7: Dough formulas for preparation of sourdoughs and aseptic control doughs

	neutral control	**acid control**	***L. pontis***	***L. sanfr.***	**acid control + protease**	***L. pontis* + protease**
Wheat flour (mg)	400	400	400	400	400	400
Tap water (µL)	400	300	200	200	200	100
Microorganism (µL)[b)]	-	-	200	200	-	200
Chloramphenicol 10 % w/ vol (µg)	2	2	-	-	2	-
FTIC-Protein or water (µL)	100	100	100	100	100	100
Protease (mg) [a)]	-	-	-	-	1.7	1.7
Acid (µL) [a)]	-	100	-	-	100	-

[a)] Mixture of 4 volumes of lactic acid (90%) and 1 volume of acetic acid (98%) in tap water to obtain a dough pH of 3.5

[b)] Approximately 10^9 cells of *L. pontis* TMW 1.397 and *L. sanfranciscensis* LTH2581, respectively.

[c)] Fungal Protease commercially available for use in baking improvers

2.7 Amino Acid Analysis from Food by IPAD

2.7.1 Chemicals and reagents

The amino acid standard contained all proteinogenic amino acids and additionally ornithine and cystine. The concentration of cystine was 0.21 mM, all other amino acids were added at a concentration of 0.42 mM. The complete standard was prepared from a standard reference material (17 amino acids at a concentration of 2.5 mM dissolved in 0.1 N HCl) obtained by Sigma Aldrich (St. Louis, MO) and the following amino acids (all from Sigma and p.a. grade) were prepared as 2.5 mM single component stock solutions in 0.1 N HCl and added afterwards: asparagine, glutamine, cysteine, ornithine, tryptophan.

All sugars and sugar alcohols were purchased as solids from Sigma Aldrich at p.a. grade. The stock solutions were prepared by dissolving the analytes in 0.1 N HCl at a concentration of 2.5 mM, and a standard containing glucose, fructose, sucrose, and erythritol was obtained by mixing the stock solutions in equimolar amounts.

All solvents and eluents were prepared with water with a resistance of 18 megaohm or more was freshly prepared with a facility from SG-water (Barsbuettel, Germany). Three different Eluents (A, water; B, 250 mM NaOH; C, 1 M sodium acetate) were essentially prepared according to the supplier (Anonymous, 2000) using a 50 % NaOH solution (J. D. Baker, Philipsburg, NJ) and anhydrous sodium acetate (Fluka, Deisenhofen, Germany). All eluents were exhaustively degassed with helium and kept under slight helium overpressure.

For solid phase extraction 1 ml tubes (Strata SCX, Phenomenex, Torrance, USA) containing 100 mg sorbent mass were used. Eluents were prepared with 18 megaohm water, methanol, calcium chloride dihydrate and hydrochloric acid 37% (all chemicals Merck, Darmstadt, Germany and p.a. grade). Food samples were precipitated using 7 % perchloric acid diluted from a 70% solution (Riedel-de-Haen, Deisenhofen, Germany, ACS grade).

2.7.2 Liquid chromatography

A HPLC consisting of a M480 pump, autosampler, and column thermostat (all components Gynkothek, Germering, Germany) coupled to a ED40 electrochemical detector containing a gold working electrode and an pH reference electrode (Dionex, Sunnyvale, USA) was used. The chromatographic system control as well as the data acquisition and the data evaluation were done with a Chromeleon 4.1 software. The separation was performed using the AminoPac PA10 column set from Dionex (Sunnyvale, USA), consisting of a guard column and a analytical column (2 mm x 250 mm). To remove carbonate the eluents were pretreated with ATC1 columns, one for each eluent line, and metal ions were captured using an MFC1 column installed between pump and Rheodyne valve (all columns from Dionex, Sunnyvale, USA).

Table 2.8: Modified HPLC gradient for samples containing high ion concentrations

Time (min)	**% solvent A**	**% solvent B**	**% solvent C**	
-40.0	0	50	50	Column wash
-30.0	0	50	50	
-29.9	84	0	16	Column equilibration
0.0	84	0	16	Inject, acquisition on
2.0	84	0	16	
10	68	0	32	
16	60	0	40	
20	36	40	24	
37	36	40	24	
45.0	0	50	50	Acquisition off

The detection of sugars and amino acids was performed with one wave form and two different integration periods described in table 2.9 (Jandik et al., 1999).

To separate the metabolites a ternary gradient shown in table 2.8 was used. The ternary gradient is based on the recommendation by the supplier (Anonymous, 2000) and was optimized for samples containing high ion concentrations. The separation was performed at a column temperature of 30 °C and at a flow rate of 0.25 ml/min.

Table 2.9: Detection waveforms for amino acid and sugar detection according to Jandik et al. (1999)

Time (ms)	**Potential (V) vs. pH electrode**	**Integration**
0	+0.13	
40	+0.13	
50	+0.33	
110	+0.33	Start sugar (Period 1)
210	+0.33	End sugar, start amino acid (Period 2)
220	+0.60	
460	+0.60	
470	+0.33	
560	+0.33	End amino acid
570	-1.67	
580	-1.67	
590	+0.93	
600	+0.13	

Period 1 is used both for amino acids and sugars and period 2 is specific for sugars, only a few amino acids, e.g. proline, show a minor signal.

2.7.3 Solid Phase Extraction

Method 1 (Thurman and Mills, 1998) One part amino acid standard was diluted with 1 part methanol and 8 parts of water. The cation exchange sorbent was washed with 3 column volumes methanol to wet the resin, and the methanol was subsequently removed with 3 column volumes water. 1 ml of sample was applied by gravity feed to the column. The samples were eluted with 1 ml of 0.1 N HCl.

Method 2 (Anonymous, 1998). One part amino acid standard was diluted with 9 parts water and the pH was adjusted to pH 7.0. The columns were conditioned with 1 ml each of n-hexane, methanol and water. 1 ml sample was applied to the column by vacuum (100 mbar). After washing the column with

1 ml water the column was dried under vacuum for three minutes. The amino acids were eluted with 1.5 ml of 0.1 N HCl.

Method optimization. The column was washed with 3 ml methanol and 3 ml water. The standard was diluted 2 to 10 times with 10% methanol and 0.01 to 0.05 N HCl or with 0.01 % HCl and 0% to 50% methanol. 1 or 2 ml of the sample were added and allowed to pass by gravity. The column was washed with 1 ml HCL-methanol mixture of the former used concentration. The amino acids were eluted with varying amounts of $CaCl_2$ and HCl solutions of different concentrations.

Method 3. Based on the method optimization, the following method was used for all food samples. The column was conditioned with methanol and water. Samples were diluted 10 fold with 0.01 N HCl and 10% methanol and 1 or 2 mL depending on the amino acid content in the sample was applied by gravity. The column was washed with 1 ml water and amino acids were eluted with 2 ml 0.2 M CaCl2 and 1 ml demineralized water. Prior to HPLC determination a at least 5-fold dilution for amino acids and an appropriate dilution for sugar was prepared.

Extraction of food samples. Sourdough was prepared as described (Thiele et al., 2002), other samples were obtained at a local supermarket. Samples were diluted with water to an approximately solid to fluid ratio (after centrifugation) of 1 to 5 or more. If de-proteinization was desired, perchloric acid was added to a final concentration of 3.5%. Upon thorough mixing, the samples were centrifuged and the supernatants were used for solid phase extraction.

2.7.4 Recovery of amino acids from food samples.

Amino acids from sourdough and lemon juice were extracted and separated from carbohydrates, and their concentrations were determined by HPLC. Norleucine was added to the samples prior to sample preparation at a level of 25 μM as internal standard. In order to exclude incomplete recoveries of individual amino acids from foods, the experiment was repeated and the amino acid standard solution was added to the food samples prior to sample preparation to increase the amino acid concentrations by 25μM. The amino acid concentrations of sourdough and lemon juice with amino acids internal standard was determined by HPLC. The differences between amino acid concentrations in the absence and presence of amino acids internal standard were used to calculate recoveries of individual amino

acids. The experiments were carried out in triplicate. Amino acid recoveries were not calculated if the amino acid concentration in the food sample exceeded the amino acid levels originating from the internal standard more than 10fold.

3. Results

3.1 Contribution of microorganisms and wheat enzymes on the flavor of rolls

The contribution of cereal and microbial enzymes to the proteolytic liberation of amino acids during the sourdough fermentation as well as their relevance for sensory attributes of bread was determined. The effect of acidification and reducing agents on proteolysis in sterile wheat doughs was compared to the proteolytic activities of sourdoughs fermented with defined starter cultures. To assess the impact of proteolysis on bread flavor, the concentrations of individual amino acids relevant as precursor compounds for flavor volatiles – ornithine, phenylalanine, leucine, isoleucine, and methionine – was determined, and baking experiments were carried out using sterile or lactic fermented doughs with known content of amino acids. The relevance of the dough amino acid levels for bread flavor was determined by the sensory analysis of bread.

3.1.1 Effect of salt, acid and dithiothreitol (DTT) on the total amino nitrogen content in sterile wheat doughs.

To take into account pH and rH effects on the activity of wheat flour proteolytic enzymes during the sourdough fermentation, the amino nitrogen release in sterile doughs was determined. Wheat doughs with different additives were incubated for 3 days at 30°C. Microbial growth was inhibited by addition of erythromycin, chloramphenicol and cycloheximide and cell counts of sterile doughs did not exceed 10^4 cfu/g, excluding an influence of microbial proteases. The amino nitrogen concentrations of sterile wheat doughs are shown in figure 3.1.

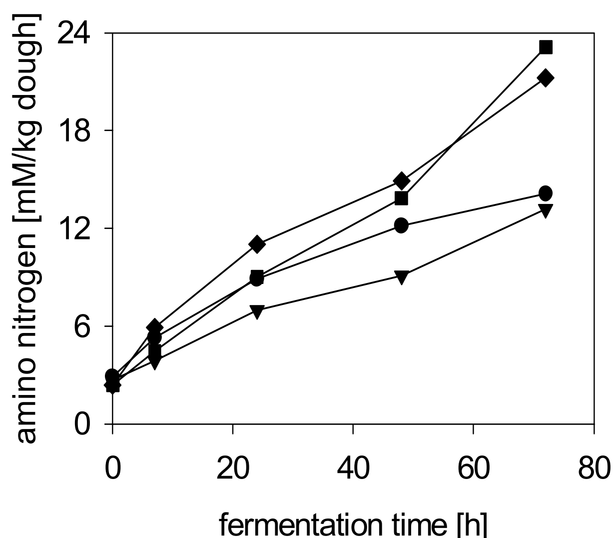

Figure 3.1. Concentration of total amino nitrogen in sterile fermented doughs. Shown is the amino nitrogen concentration in dough without additives (▼, n=4), dough containing 4% NaCl (●, n=2), dough acidified to pH 3.8 (■, n=4), and dough containing 0.7% DTT (◆, n=2). Shown are mean values of n fermentations and the coefficient of variation was less than 10 %.

In all doughs the amino nitrogen content increased during fermentation. After 24 hours between 4,3 mM and 8,3 mM amino nitrogen is released. The addition of salt reduced the release of amino nitrogen. In contrast, addition of lactic and acetic acids and DTT increased the rate of proteolysis. Whereas after 24 h the highest amino nitrogen levels were found in sterile, DTT doughs, amino nitrogen levels of reduced and acidified doughs were virtually identical after 72 h of incubation.

3.1.2 Total amino nitrogen content in fermented wheat doughs

Wheat doughs were fermented with the following starter cultures: *Saccharomyces cerevisiae*, *Candida milleri*, *Lactobacillus sanfranciscensis*, *Lactobacillus pontis* and mixtures of *L. pontis* with *S. cerevisiae* or *C. milleri*. Because the demand of microorganisms for amino acids during growth as well as the pH drop during fermentation are likely to affect amino acid concentrations, the growth of microorganisms as well as the dough pH were determined. The growth of the microorganisms in doughs is shown in figure 3.2.

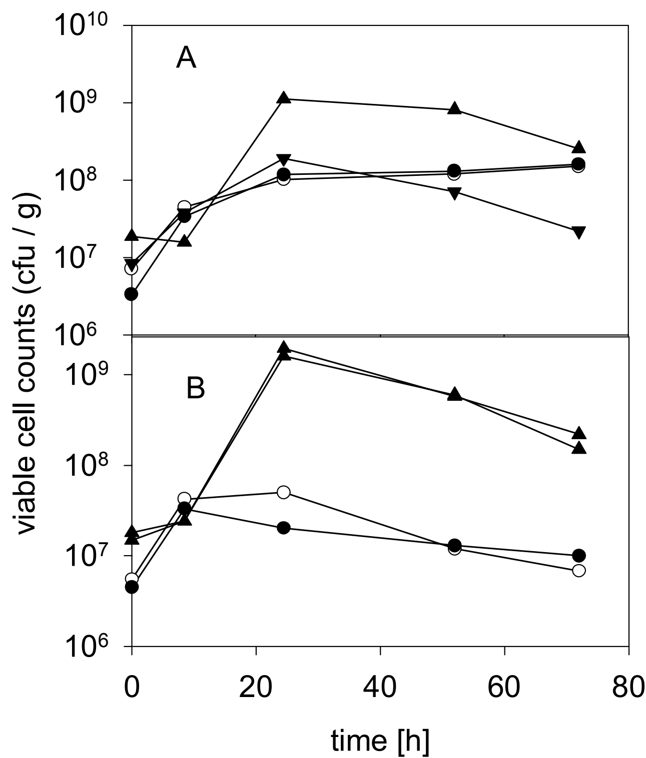

Figure 3.2. Cell counts of lactic acid bacteria and yeasts during dough fermentation. Shown are the cell counts of pure cultures (A) and mixed cultures of *L. pontis* and *S. cerevisiae* or *C. milleri* (B). Symbols indicate cell counts of *L. sanfranciscensis* (▼), *L. pontis* (▲), *S. cerevisiae* (❍), and *C. milleri* (●). Data are representative for duplicate fermentations.

In all fermentations, contaminants were not detectable, i.e. they accounted for less than 0.1% of the total viable cell counts. The yeasts and *L. sanfranciscensis* started to grow essentially without lag and reached the stationary growth phase after 24 h of incubation. *L. pontis* exhibited a lag phase of about 4 h and grew exponentially between 10 – 20 h. The cell counts of lactic acid bacteria decreased by about one order of magnitude in the stationary phase of growth. Whereas cell counts of *L. pontis* were unaffected by the presence or absence of yeasts, the cell counts of *S. cerevisiae* and *C. milleri* were considerably reduced in the presence of *L. pontis*.

The pH-values of fermented doughs are shown in figure 3.3.

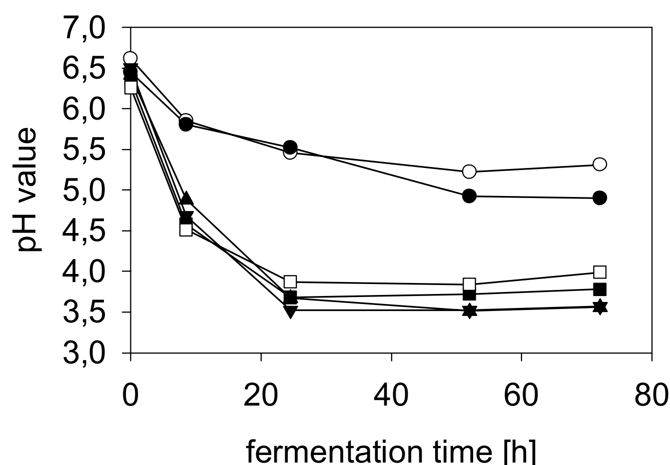

Figure 3.3. Evolution of pH in doughs fermented with lactobacilli and yeasts. Shown is the pH value of doughs fermented with *S. cerevisiae* (❍), *C. milleri* (●), *L. sanfranciscensis* (▼), *L. pontis* (▲), *L. pontis* and *S. cerevisiae* (❐), and *L. pontis* and *C. milleri* (■). Data are representative for duplicate fermentations

In doughs fermented with lactobacilli the pH decreases from about 6.5 to 4.5-5.0 within 8 hours. After 24 hours, when the lactobacilli reached the stationary growth phase, acid production ceased and the pH remained stable. The final pH of doughs fermented with lactobacilli only was about 0.2 units lower than the pH of doughs fermented with mixed cultures of yeasts and lactobacilli. In doughs fermented with pure cultures of *S. cerevisiae* and *C. milleri*, the pH decreased to 5.3 and 4.9, respectively, within 52 h and remained stable afterwards.

The total amino nitrogen of fermented doughs during fermentation is shown in figure 3.4.

The release of amino nitrogen is clearly related to the growth of the organisms. In all doughs fermented with yeasts, a decrease in amino acid concentration during the exponential growth phase was detected. Amino acid concentrations started to increase after the yeasts entered the stationary phase of growth. In doughs fermented with lactobacilli only, amino acid concentrations linearly increased throughout the fermentation. The final amino acid concentration was highest in doughs fermented with *L. pontis*, independent of the presence or absence of yeasts. Fermentation with *L. sanfranciscensis* resulted in slightly lower amino acid concentrations. The lowest amino nitrogen concentrations were found in doughs fermented with yeasts only. Whereas 20 mmol kg^{-1} amino nitrogen were detected in doughs fermented with *L. pontis*, only 14 and 10 mmol kg^{-1} were determined in doughs fermented with *C. milleri* and *S. cerevisiae*, respectively.

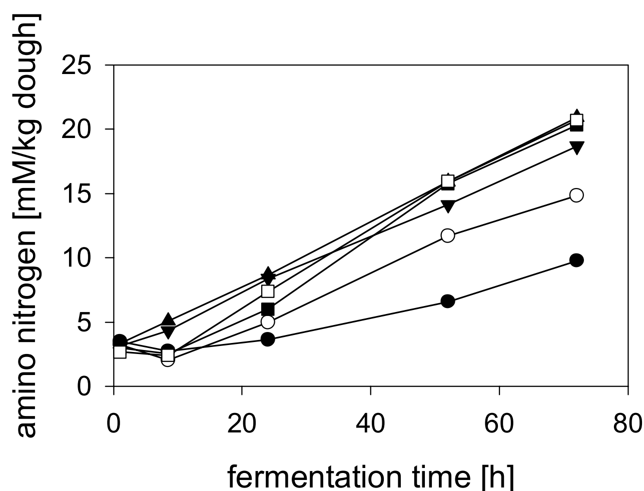

Figure 3.4. Concentration of total amino nitrogen in doughs fermented with lactobacilli and yeasts. Shown is the amino nitrogen concentration in dough fermented with *S. cerevisiae* (❍), *C. milleri* (●), *L. sanfranciscensis* (▼), *L. pontis* (▲), *L. pontis* and *S. cerevisiae* (❒), and *L. pontis* and *C. milleri* (■).Shown are mean values of duplicate fermentations and the coefficient of variation was less than 10 %.

A comparison of sterile fermented doughs with those doughs inoculated with lactobacilli or yeasts allows to estimate the contribution of microbial proteases to proteolysis in wheat doughs. In doughs fermented with yeasts, the amino nitrogen concentration during the first 24 h was lower than that of any sterile doughs, indicating that the amino acid consumption of yeasts is greater than any proteolytic activity the organisms may exhibit. The release of amino nitrogen in doughs fermented with lactobacilli did not exceed the amino nitrogen concentrations in acidified or reduced doughs, indicating that the proteolytic activity of lactobacilli is neglectible compared to the proteolytic activity of wheat flour. The reduction of the pH and rH value brought about by the lactic fermentation however, greatly enhanced proteolysis in dough compared to neutral, sterile doughs.

3.1.3 Changes in individual amino acid concentration

The concentration in dough of the following amino acids is of prime importance for bread flavor: ornithine, methionine, phenylalanine, leucine, isoleucine, and valine. We therefore determined the concentration of individual amino acids in dough by HPLC. In order to compare the amino acid composition of doughs with different absolute levels of total amino acid concentration, the content of individual amino acids relative to the total amino acid concentration was calculated. The amino acid concentration determined by HPLC agreed well with the total amino nitrogen determined with the ninhydrin method (r^2 = 0.95 or greater). The relative contents of sterile and fermented doughs of arginine and ornithine are shown in Figures 3.5A and 3.5B, respectively.

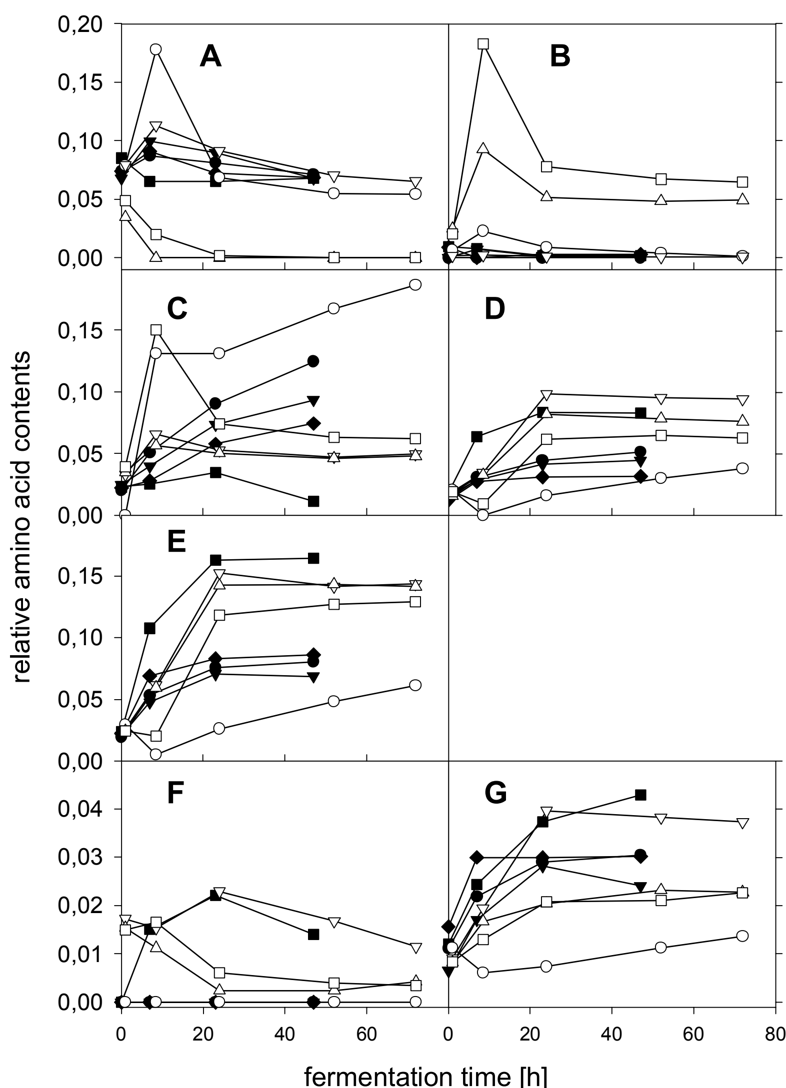

Figure 3.5. Relative amino acid contents of sterile and fermented doughs during incubation at 30°C. Shown is the concentration of arginine (A), ornithine (B), proline (C), phenylalanine (D), leucine (E), cysteine (F) and methionine (G) relative to the total amino acid concentration as determined by HPLC. Symbols indicate sterile doughs without additives (●), sterile dough with 4% NaCl (▼), sterile dough acidified to pH 3.5 (■), sterile dough with 0,7% DTT (◆), dough fermented with *S. cerevisiae* (❍), *L. sanfranciscensis* (∇), *L. pontis* (Δ) and *L. pontis* and *S. cerevisiae* (❒). Shown are mean values of duplicate fermentations and the coefficient of variation was less than 5 %.

Because ornithine, one of the precursors for acetylpyrroline, is not a proteinogenic amino acid, its presence in dough is the result of microbial metabolism. Ornithine was found only in doughs fermented with *L. pontis*. Arginine was released by proteolytic enzymes from wheat proteins and the relative content of arginine was largely independent of the presence of acid, DTT or salt. Arginine was

not found in doughs fermented with *L. pontis*, however, the ornithine levels in these doughs corresponded well to the arginine levels in sterile doughs or doughs fermented with *L. sanfranciscensis* or yeasts.

The relative proline content of sterile and fermented doughs is shown in Figure 3.5C. The highest proline contents were found in yeasted doughs and sterile neutral doughs. In contrast, relative proline contents remained low in sterile acidified doughs. In doughs fermented with lactobacilli, proline levels corresponded to those of neutral doughs in the first hours of fermentation and a decrease of the relative proline content was observed after the dough pH was lowered to 5.5 or less. After 72 hours, the relative content of proline was less than 1/3 of the neutral and yeasted doughs. These results show that the release of proline depends strongly on the dough pH. The high relative proline content of doughs fermented with *S. cerevisiae* indicates that proline, in contrast to most other amino acids, is not metabolized by the yeast.

The relative levels of phenylalanine and leucine in sterile and fermented doughs are shown in Figures 3.5D and 3.5E, respectively. The release of these amino acids was low in sterile neutral doughs and the highest levels of hydrophobic amino acids were found in chemically acidified and lactic fermented doughs. Both amino acids were metabolized by *S. cerevisiae* during exponential growth. *L. sanfranciscensis* and *L. pontis* did not exhibit any demand for these amino acids. During the first 10 hours the relative content of phenylalanine and leucine compared well to that of neutral sterile doughs and when the dough reached a pH of about 4.0, phenylalanine and leucine levels were comparable to that of sterile acidified dough.

Cysteine affects dough rheology and methionine serves as precursor for the aroma compound 3-(Methylthio)propanal. The relative contents of cysteine and methionine of sterile and fermented doughs are shown in Figures 3.5F and 3.5G, respectively. Cysteine was released only in acidified doughs, in all other sterile samples only traces were detected. Likewise, methionine levels in sterile acidified doughs were about 50% higher than that of neutral doughs. Whereas cysteine and methionine levels of doughs fermented with *L. sanfranciscensis* were comparable to that of chemically acidified doughs, fermentation of doughs with *L. pontis* reduced the levels of both amino acids. This indicates that *L. pontis*, in contrast to *L. sanfranciscensis* degrades these amino acids.

3.1.4 Sensory evaluation of rolls

To establish the influence of different preferments on the taste of wheat bread rolls baking experiments and subsequent flavor analysis were carried out. The results of the descriptive sensory evaluation and the ranking according to the aroma intensity are given in Table 3.1.

Table 3.1: Sensory analysis of wheat rolls. The doughs were supplemented with different preferments as indicated in Table 2.3 and doughs within a row were compared with each other and ranked according to their aroma intensity.

	aroma intensity		
lowest	low	high	highest
D1: mild, flat	D3 aromatic, bitter, burnt, cheesy, rubber-like		
D1, D2: mild, flat	D4: not fresh, slightly more aromatic, slightly roasty	D5, D6: more aromatic, yeasty, mild sour	
D1: mild, flat	D7: more aromatic, mild sour, slightly fermented	D8: more aromatic, well rounded	D9: aromatic, roasty to burnt, bitter, sour
D1: mild, flat	D10: slightly aromatic, slightly roasty	D11: aromatic, roasty, mild sour	
D1: mild, flat	D12: slightly sour, mild	D13: aromatic very sour, very roasty	

A total of three different preferments was prepared on a single day and compared with a control roll without addition of a preferment. Any bread prepared with a preferment had a more aromatic flavor than the control bread.

Doughs D1 to D3 were prepared to evaluate the effect of addition of amino acids to bread dough on bread flavor. Addition of ornithine, isoleucine, leucine, phenylalanine, and methionine at concentrations comparable to microbial preferments did not result in significant differences compared to the control bread. When 10 mmol amino acids per kg flour were added, an aromatic and roasty to slightly burnt odor was detectable. The taste was bitter, burnt, slightly cheesy and like rubber. Preferments inoculated with either *S. cerevisiae* or *C. milleri* improved the flavor of the rolls; compared to the controls, a more aromatic and yeasty flavor was perceived (doughs D5 and D6).

Chemically acidified preferments produced breads with a slightly roastier flavor than the control breads. In comparison, doughs fermented with *L. sanfranciscensis* exhibited an improved aroma despite comparable levels of acidity as well as organic acid and amino acids concentrations. When ornithine was additionally added to the *L. sanfranciscensis* preferment before baking, the overall impression of the flavor improved. The flavor of breads prepared with preferments containing *L. sanfranciscensis* and *L. pontis* were clearly discernible by the more roasty flavor of the latter breads. Addition of 13% *L. pontis* preferment was perceived as “burnt” and “bitter” by several panelists.

To distinguish between the effect of microbial metabolism and amino acid accumulation, preferments with *L. pontis* were incubated for 20 and 40 h. After 20h, growth and organic acid production by *L. pontis* ceased but proteolytic release of amino acids and ornithine formation by *L. pontis* continued over 40 h. The roasty note of bread crust was enhanced by increased fermentation times of the preferments. The flavor intensity of the 40 h fermented bread was so high that it was perceived as unpleasant by several panelists. The roasty note of bread could further be enhanced by increasing levels of *L. pontis* preferments added to the bread doughs (doughs D11, D12, and D13).

3.2 Screening of proteolytic active lactic acid bacteria

The release of amino acid during the sourdough fermentation is increasing the amount of potential precursors of wheat bread flavor. For a further improvement of flavor lactic acid bacteria were screened on their proteolytic activity on gluten and casein. Agar plates were in a first step made according to Wiese (1995) for gluten plates and according to Frazier and Rupp (1979) for calcium-caseinate plates. On both plate types no growth of lactic acid bacteria was detectable, only *B. subtilis* which was used as positive control grew. In a second step media were modified with respect to sugars, yeast extract, vitamins and trace elements. The maximum amount of yeast extract was restricted to 2.5 g per liter to avoid abundance of easy accessible nitrogen compounds. Growth of lactic acid bacteria on proteolytic screening plates is shown in table 3.2, all growth experiments were performed in quadruplicate. All micro-organisms were able to grow on Homiochii plates, growth of most bacteria on screening plates was meager but detectable. None of the used lactic acid bacteria strains exhibited proteolytic activity on agar plates was detectable, only *B. subtilis* showed clearance of agar plates.

Table 3.2: Lactic acid bacteria used for screening on proteolytic activity. Growth on Gluten-Agar and Ca-Caseinate-Agar was determined, growth was marked with x, no growth with n.g.. All strains were able to grow on Homiochii plates.

TMW number	*Strain*	Gluten Agar	Ca-Cas. Agar
TMW 1.8	*Lactobacillus fructosus (Leuconostoc)*	n.g.	x
TMW 1.9	*Lactobacillus plantarum*	x	x
TMW 1.10	*Lactobacillus pentosus*	x	x
TMW 1.11	*Lactobacillus alimentarius*	x.	x.
TMW 1.12	*Lactobacillus pentosus*	x	x
TMW 1.17	*Lactobacillus curvatus*	x.	n.g.
TMW 1.18	*Lactobacillus acidophilus* LTH 1220	n.g.	x.
TMW 1.19	*Lactobacillus animalis*	x.	x.
TMW 1.20	*Lactobacillus kandleri*	x.	x.
TMW 1.25	*Lactobacillus cerealis*	x	x
TMW 1.28	*Lactobacillus pentosus*	x	n.g.
TMW 1.29	*Lactobacillus plantarum*	x n	x
TMW 1.52	*Lactobacillus sanfranciscensis* LTH 2581	x	x
TMW 1.53	*Lactobacillus sanfranciscensis* ATTC 27651	x	x
TMW 1.54	*Lactobacillus cerealis or sanfrancisco*	x	x
TMW 1.55	*Lactobacillus alimentarius*	x	x
TMW 1.58	*Lactobacillus delbrückii ssp. delbrückii*	x.	x.
TMW 1.59	*Lactobacillus fructivorans*	x.	x.
TMW 1.60	*Lactobacillus plantarum* DSM 20174	x.	x.
TMW 1.63	*Lactobacillus rhamnosus*	x.	x.
TMW 1.66	*Lactobacillus delbrückii ssp. Lactis* DSM20072	x.	x.
TMW 1.67	*Lactobacillus casei ssp. casei* DSM 20011	x.	x.
TMW 1.68	*Lactobacillus farciminis*	x.	x.
TMW 1.70	*Lactobacillus viridescens*	x.	x.
TMW 1.72	*Lactobacillus delbrückii ssp bulgaricus* DSM 20081	x.	x.
TMW 1.80	*Lactobacillus spec.*	x.	x.
TMW 1.81	*Lactobacillus reuteri*	x	x
TMW 1.83	*Lactobacillus pontis*	n.g.	n.g.
TMW 1.84	*Lactobacillus pontis*	x	x
TMW 1.85	*Lactobacillus pontis*	x	x

TMW 1.100	*Lactobacillus brevis*	x	x
TMW 1.101	*Weissella confusa*	x	x
TMW 1.119	*Lactobacillus brevis*	n.g.	x.
TMW 1.126	*Lactobacillus manihotivorans*	n.g.	n.g.
TMW 1.188	*Lactobacillus amylovorus*	x	x
TMW 1.397	*Lactobacillus pontis* LTH 2587	x	x
TMW 1.402	*Lactobacillus sakei*	n.g.	x.
TMW 1.460	*Lactobacillus brevis*	x	x
TMW 1.461	*Lactobacillus paracasei ssp. paracasei*	x.	x.
TMW 1.487	*Lactobacillus amylolyticus*	x	x
TMW 1.509	*Lactobacillus paracasei*	x.	x.
TMW 1.511	*Lactobacillus buchneri*	n.g.	x.
TMW 1.535	*Lactobacillus plantarum*	x	x
TMW 1.541	*Lactobacillus spec.*	x	x
TMW 1.557	*Lactobacillus spec.*	x	x
TMW 1.558	*Lactobacillus spec.*	x	x
TMW 1.613	*Lactobacillus fermentum*	x	x
TMW 1.616	*Lactobacillus sanfranciscensis*	x	x
TMW 1.617	*Lactobacillus sanfranciscensis*	x	x
TMW 1.648	*Lactobacillus panis* DSM 6035	x.	x.
TMW 1.649	*Lactobacillus panis*	x	x
TMW 1.653	*Lactobacillus amylovorus*	x	x
TMW 1.655	*Lactobacillus farciminis*	x	x
TMW 1.666	*Lactobacillus sanfranciscensis*	x	x
TMW 1.675	*Lactobacillus pontis*	x	x
TMW 1.682	*Lactobacillus pontis*	x	x
TMW 1.693	*Lactobacillus reuteri*	x.	x.
TMW 1.695	*Lactobacillus helveticus*	n.g.	x.
TMW 1.724	*Lactobacillus sanfranciscensis*	x.	x.
TMW 1.725	*Lactobacillus sanfranciscensis*	x.	n.g.
TMW 1.725	*Lactobacillus sanfranciscensis*	x	x
TMW 1.726	*Lactobacillus sanfranciscensis*	n.g.	x.
TMW 1.727	*Lactobacillus sanfranciscensis*	n.g.	x.
TMW 1.837	*Lactobacillus plantarum*	x	x
TMW 1.838	*Leuconostoc mesenteroides*	x	x
TMW 1.890	*Lactobacillus fermentum*	x	x

TMW 1.895	*Lactobacillus cerealis*	x	x
TMW 1.923	*Pediococcus pentosaceus*	x	x
TMW 1.931	*Enterococcus faecium*	x	x
TMW 1.934	*Weissella confusa*	x	x
TMW 1.939	*Lactobacillus brevis*	x	x
TMW 1.947	*Lactobacillus alimentarius*	x	x
TMW 1.949	*Lactobacillus sanfranciscensis*	x	n.g.
TMW 1.953	*Lactobacillus sanfranciscensis*	x	x
TMW 1.977	*Lactobacillus reuteri*	x	x
TMW 1.1009	*Lactobacillus pontis*	x	x
TMW 1.1078	*Lactobacillus amylolyticus*	x	x
TMW 1.1085	*Lactococcus lactis*	n.g.	n.g.
TMW 1.1109	*Lactobacillus acidophilus*	x	x
TMW 1.1112	*Lactobacillus pontis* HMSD In1	n.g.	n.g.
TMW 1.1120	*Lactobacillus panis*	x	x
TMW 1.1143	*Lactobacillus oris*	n.g.	x.

The missing proteolytic degradation of insoluble proteins by lactic acid bacteria may be a result of a screening system which is not sensitive enough for the not very potent cell wall bound proteases. A very sensitive test system based on the degradation of fluorescence labeled proteins was used with two different strains of *L. sanfranciscensis*. Casein and wheat protein extracts were labeled with FITC (Twining, 1984; Lindsay et al., 2000) and incubated with proteases and microorganisms for several hours. Fluorescence was measured online, depolymerization of proteins results in an increase of fluorescence due to release of quenching effects (Farmer and Yuan, 1991). In figure 3.6 relative fluorescence over time is shown.

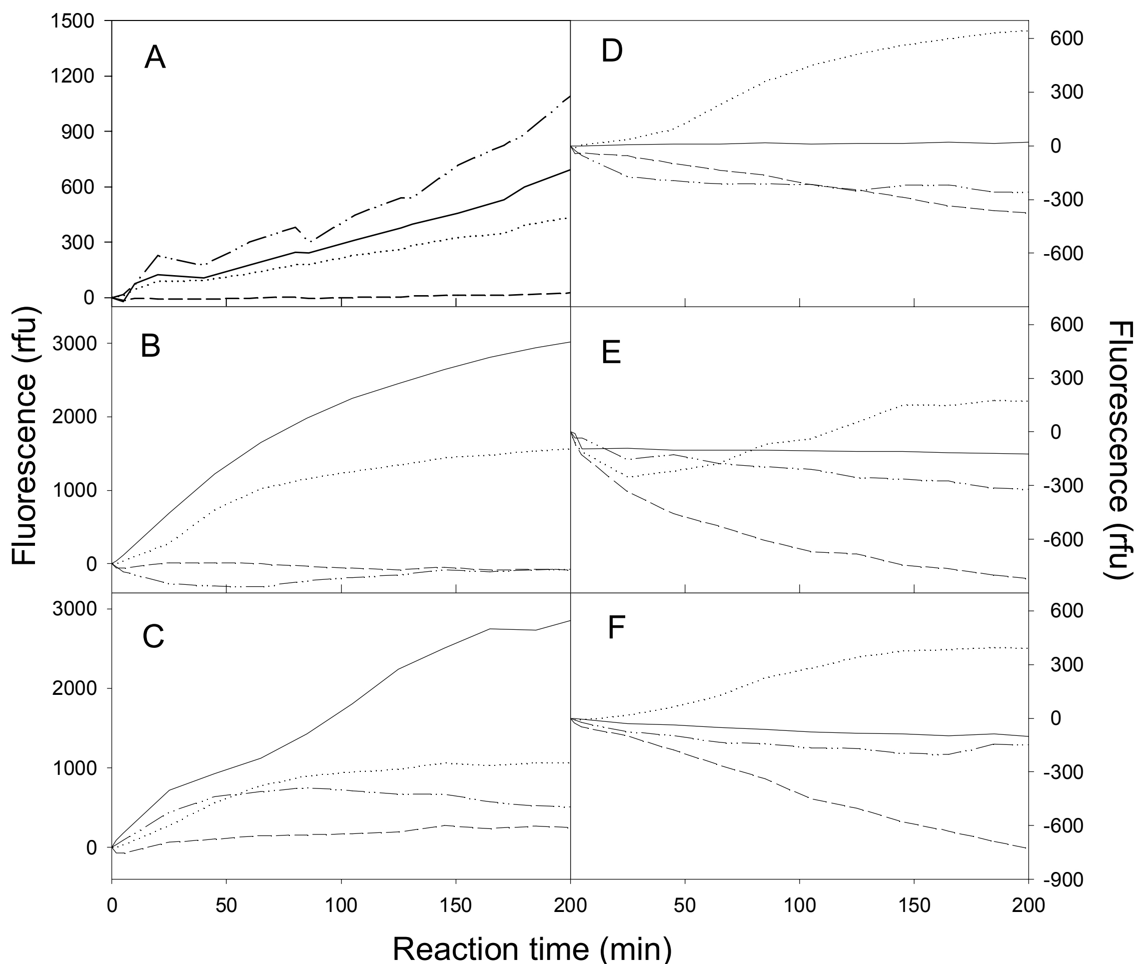

Figure 3.6: Online-detection of proteolytic degradation using FITC-labeled proteins. Samples were incubated for 200 min at 30°C in the Tecan Spectrafluor, fluorescence was detected at Em/Ex 485 nm and 520 nm. The fluorescence traces represent: Figure A: Hydrolysis of FITC-Casein by *B. subtilis* at pH 7.0 (solid line), 6.0 (dotted line), 5.0 (dashed line), unbuffered (dotted/dashed line). Figure B: hydrolysis by a fungal protease of: FITC-Casein (solid line), FITC-albumin/globulin (dotted line), FITC-gliadin (dashed line) and FITC-glutenin (dashed-dotted line). Figure C: hydrolysis of proteins (see B) by papain. Figure D: Auto-hydrolyzation of proteins (see B) without addition of enzymes and micro-organisms. Figure E: Hydrolysis of proteins (see B) by *L. sanfranciscensis* LTH 2581. Figure F: Hydrolysis of proteins (see B) by *L. sanfranciscensis* ATTC. Fluorescence at time 0 was set to 0 rfu for all samples.

B. subtilis (A), a fungal protease (B), and papain (C) demonstrated, that some but not all substrates can be degraded. *B. subtilis* was only tested with FITC-Casein and a significant degradation was detected, within 3 hours the fluorescence increased between 1100 and 0 rfu, depending on the pH value in the

sample. Fluorescein has its highest fluorescence at pH 8.5 and under acetic conditions fluorescence decreases rapidly. At pH 5.0 the fluorescence is too weak to detect any changes.

Proteolytic degradation of FITC-casein, FITC-albumin/globulin, FITC-gliadin and FITC-glutenin was tested with fungal protease and Papain. The data are shown for a pH of 6.0, because FITC has enough fluorescence at this pH value and lactic acid bacteria are able to grow under these conditions.

The fungal proteinase degraded only FITC-casein and FITC-albumin/globulin. Papain hydrolyzed all substrates, whereas FITC-casein and FITC-albumin/globulin showed the highest increase in fluorescence. Autohydrolysis of substrates was tested for all FITC-proteins. The fluorescence over time for pH 6.0 are shown in figure 3.6D. Proteins showed different characteristics, fluorescence of FITC-casein did not change during time. FITC albumin showed an increase in fluorescence during time while FITC-gliadin and FITC-glutenin showed an overall decrease in fluorescence. Proteolytic activity of lactobacilli was tested for two strains which are relevant in wheat sourdough fermentation. *L. sanfranciscensis* ATTC 27651 is the type strain and shows according Mitra (1999) low proteolytic activity, *L. sanfranciscensis* LTH 2581 is a strain isolated from industrial sourdough and is repeatedly found for about 20 years. For both strains only the sample with FITC-albumin/globulin showed any increase in fluorescence, but the increase of fluorescence was lower than the autolysis of FITC-albumin. For all other fluorescent protein substrates the fluorescence decreased during the experiment. Both strains did not show any proteolytic activity although the fermentation was carried on for more than 10 hours (data not shown). In experiments with a very low buffer concentration a decrease in pH was detectable during the fermentation (data not shown) the microorganisms were therefore still metabolic active.

The missing proteolytic activity was in good correlation with the screening on agar plates and therefore no more experiments were performed on proteolytic activity. Further investigations were done on peptide and amino acid utilization.

3.3 Nitrogen demand and peptide utilization by *L. sanfranciscensis*

In chapter 3.1 it was shown that growth of lactobacilli in sour dough did not decrease the concentration of amino acids. To determine how *L. sanfranciscensis* supplies the demand for nitrogen, growth experiments under nitrogen limited conditions were performed. It should be elucidated, which dough samples fractionated by different HPLC techniques enable growth of LABs. The ability of *L. sanfranciscensis* to grow on these fractions containing proteins, peptides or amino acids allow conclusions on the preferable amino nitrogen.

3.3.1 Dough extraction and preparative SEC

Three different types of wheat dough were fermented for 48 hours. One was a aseptic sterile dough and two doughs fermented either with *S. cerevisiae* and *L. pontis*. At the end of the fermentation the pH in the aseptic acidified dough and in the dough containing *L. pontis* was both 3.8, whereas the yeasted dough reached only 4.8. Doughs were extracted with TEA-buffer and acetonitrile and afterwards concentrated and separated by SEC. Samples were qualitatively and quantitatively analyzed by SEC, the chromatograms of dough extracts before the fractionation are compared in figure 3.7.

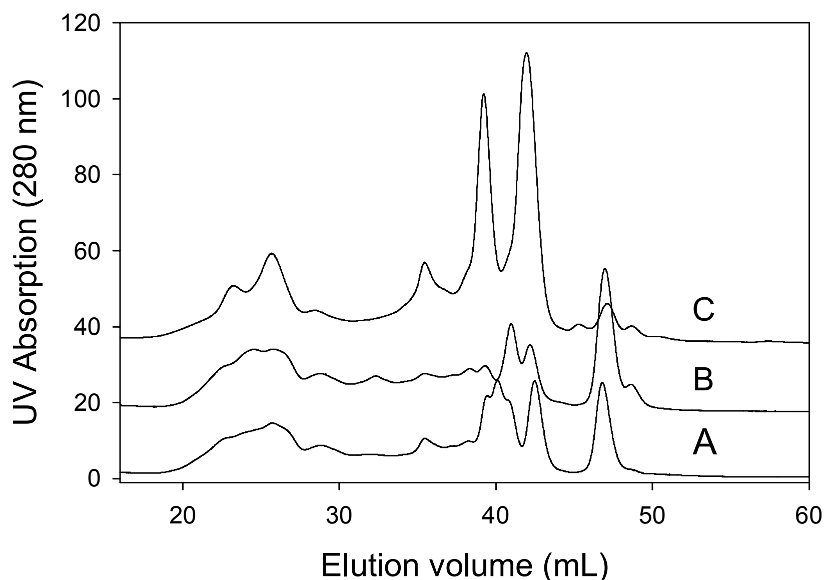

Figure 3.7: Concentrated raw-extracts from doughs for the determination of nitrogen demand of *L. sanfranciscensis*. UV-280 nm traces represent extractions after 48 hours from a aseptic sterile dough (trace A) and doughs fermented with *L. pontis* (trace B) and *S. cerevisiae* (trace C). Chromatograms are offset by 18 mAU.

Absolute concentrations between the doughs were not comparable. During sample preparation doughs were solved in the minimum amount of water therefore the dilution factor is not known. The dough fermented with L. pontis shows nearly the same size distribution at 280 nm as the aseptic acidified dough. The ratios between proteins, peptides and amino acids is about the same. Only one peak, which were eluted after about 38 ml is more dominant in the sterile dough than in the microbial fermented dough. Significant differences in size distribution are detectable between the dough fermented with yeast and the other doughs. After 38 ml two dominant peaks are detectable at a wavelength of 280 nm corresponding to aromatic compounds with a low molecular weight.

All three raw-extracts were fractionated using preparative SEC. The fractions were pooled to 4 different samples named as gel-1 to gel-4 and afterwards concentrated. The fractions were analyzed by analytical SEC and the chromatograms are shown for aseptic acidified dough in figure 3.8.

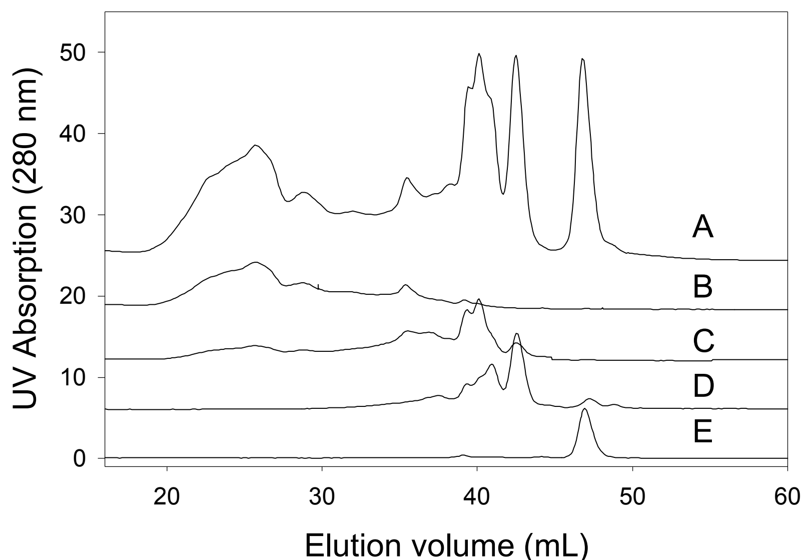

Figure 3.8: Raw-extract and fractions after SEC of an aseptic acidified dough. UV-280 nm traces represent raw extract (trace A) and fractions S-gel1 (trace B) to S-gel4 (trace E). Chromatograms are offset by 6 mAU.

The fractions contain nitrogen compounds with different size distribution. In sample S-gel 1 only proteins are detectable, fractions S-gel 2 and S-gel 3 contain peptides and amino acids, whereas S-gel 4 shows a distinct low-molecular weight peak. UV-adsorbance was detected at 280 nm. Only aromatic compounds like phenylalanine, tyrosine, and tryptophan show adsorbance and therefore all peaks should contain amino nitrogen. The area of these chromatograms was used for the adjustment of all samples to the same protein concentration determined as mAU x min.

Fractionated dough samples were tested whether they can promote growth *of L. sanfranciscensis* under nitrogen limited conditions. Growth on raw extract and fractions was determined by optical density at 590 nm, and relative growth was calculated. The growth of a culture containing 0.5 % yeast extract was defined as 100 %. All data are shown in figure 3.9.

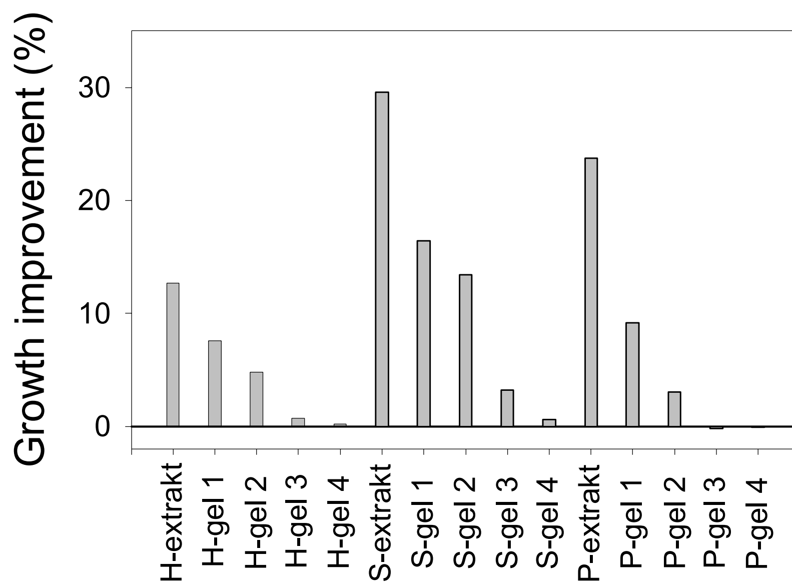

Figure 3.9: Relative growth improvement of *L. sanfranciscensis* with raw-extracts and SEC fractions from aseptic acidified dough (S) and doughs fermented with *S. cerevisiae* (H) and *L. pontis* (P). The values are calculated based on the OD measurement at 590 nm and 100 % is defined as growth on 0,5 % yeast extract.

Raw extracts from dough prior to fractionation showed highest cell density, between 15 % and 30 % of the maximal possible growth were reached. Dough fermented with yeast showed lowest and aseptic acidified doughs highest growth promotion. These differences in growth promotion can be attributed to the activation of wheat proteases by acetic pH-values of the acidified and LAB fermented doughs or due to the uptake of specific compounds by the microorganisms. After fractionation of dough extracts, growth on all dough fractions was lower compared to raw extracts. Highest values were achieved with Fraction 1 and 2, fraction 4 did not enable significant growth. The size distribution of the analytical SEC allows the conclusion that Fraction 1 contained mainly proteins (MW about 20 kDa) and peptides, and fraction 2 only peptides. The UV adsorbance in fraction 3 is mainly attributable to amino acids or other small aromatic molecules. The growth promoting compounds are therefore small proteins and peptides.

3.3.2 Dough fractionation with cation exchange chromatography

To verify that this growth activation is due to amino nitrogen, a cation exchange chromatography (CIEX) was performed. Amino acids as well as peptides and proteins are amphoteric ions, depending on the pH molecules can be charged positive or negative. Under acetic conditions most amino acids

are charged positively and therefore they interact with a negatively charged cation exchange resin. All other chemicals are eluted without interactions. Upon changing the pH to basic conditions, amino acids change their charge to neutral or negative and become eluted. All dough raw extracts were separated and the eluates were collected, dried and resolved in water. These samples were analyzed by analytical SEC to determine the size distribution and the amino nitrogen concentration. Chromatograms are shown for the aseptic acidified dough in figure 3.10.

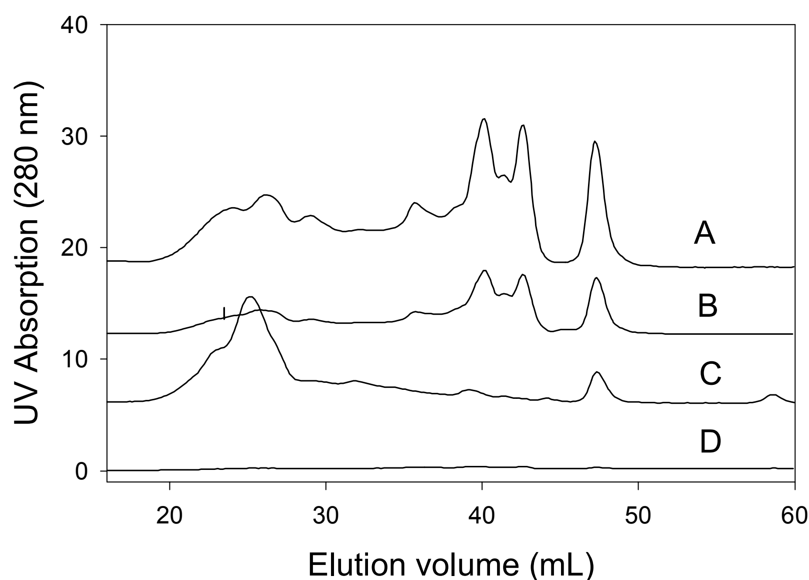

Figure 3.10: SEC of an aseptic acidified dough extract fractionated by cation exchange chromatography. The UV- 280 nm traces represent the eluate during sample application (ion-P, A), the washing step (ion-A, B), and the sequential elution of cations (ion-B1 and ion-B2, C and D).

Samples ion-P and ion-A were eluted with phosphoric acid and both show a similar size distribution. Most adsorbance is found in the low molecular weight range, but small amounts of the proteins which appear in the raw extract are found in sample ion-P as well as in sample ion-A. Samples ion-B1 and ion-B2 were both eluted with ammonia and should contain the retained cations. It is obvious that these samples show a completely different size distribution. Most adsorbance is found in the chromatogram area were proteins are expected and peptide concentration was neglectible. In sample ion-B2 almost nothing was detected, all cations seemed to be eluted in the first elution step, therefore no growth experiments with this sample were performed.

The samples were also adjusted to the same content in nitrogen determined as mAU x min and growth experiments were performed. The relative growth of *L. sanfranciscensis* on the samples is shown in figure 3.11.

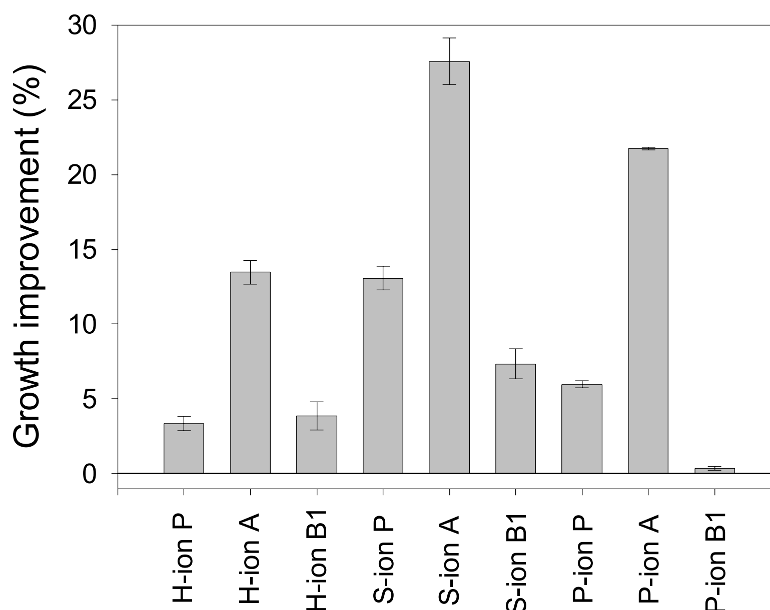

Figure 3.11: Relative growth improvement of *L. sanfranciscensis* on dough samples fractionized by CIEX from aseptic acidified dough (S) and doughs fermented with *S. cerevisiae* (H) and *L. pontis* (P). Sample values are calculated based on the OD measurement at 590 nm and 100 % is defined as growth on 0,5 % yeast extract. The values are means of 3 experiments.

Growth promotion was again similar to previous results, best growth enhancement was found with aseptic acidified incubated doughs, followed by the doughs fermented with lactobacilli and yeast. The samples ion-A achieved highest cell density within a dough. The samples H-ion P and H-ion B1 exhibited each with 5 % growth about the same improvement. In the aseptic acidified dough the sample S-ion A showed best growth promotion with 28 % , S-ion P and S-ion B1 allowed an increase in cell density to 14% and 7%, respectively. The sample P- ion P showed a growth enhancement to about 6 % whereas in sample P-ion-B no significant growth was detectable. *L. sanfranciscensis* showed again best growth on peptides.

3.3.3 Fractionation by reversed phase chromatography

For a further restriction of growth activating amino nitrogen compounds the most successful samples ion A were separated by RP chromatography. The results are shown in figure 3.12.

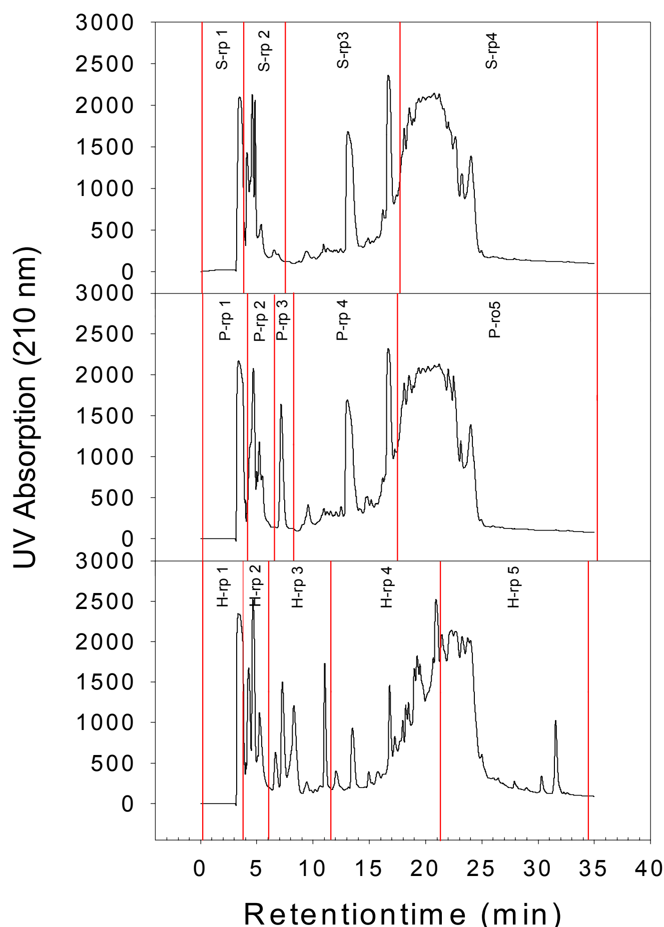

Figure 3.12: RP-Chromatography and fractionation of dough fractions derived by acetic elution during CIEX. The UV 210 traces represent the separation of H-ion A (figure A), S-ion A (figure B) and P-ion A (figure C). Collected fractions are marked within the chromatogram.

Sample H-ion A (acidic washing sample of CIEX of a dough fermented with yeast) showed peaks all over the chromatogram area, hydrophilic as well as hydrophobic peptides were present and most peptides were eluted from 18 to 25 minutes retention time. In samples S-ion A and P-ion A less hydrophilic peptides were found and most peptides show hydrophobic characteristics. These two samples show similar chromatograms, but in the sample derived from the dough fermentation with *L. pontis* one additional peak after 7.5 minutes occurred. Fermented doughs were collected in 5 different fractions (H-rp 0 to H-rp 4 and P-rp 0 to P-rp4), the sterile acidified dough was collected in 4 fractions (S-rp 0 to S-rp3). Nitrogen content was adjusted in all fractions to the same concentration determined by UV adsorbance at 210 nm. All samples were tested on their ability to improve growth. The relative growth is shown in figure 3.13.

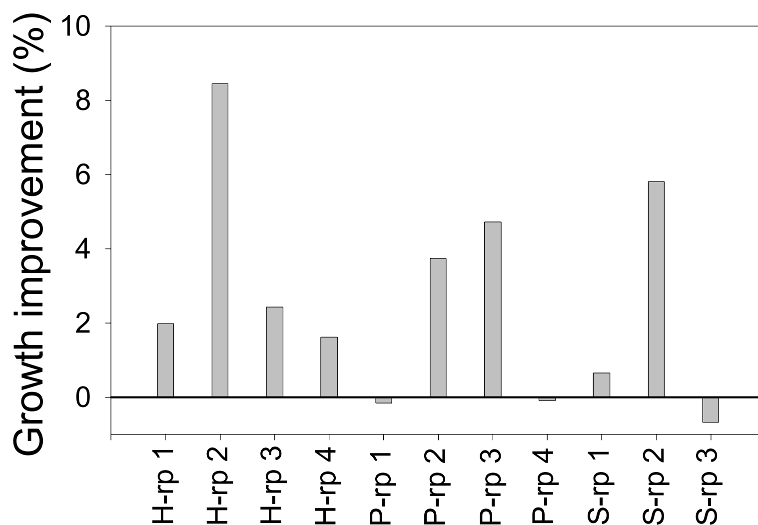

Figure 3.13: Relative growth improvement of *L. sanfranciscensis* on samples fractionized by RPC from samples derived by the cationic washing step of the CIEX of doughs fermented aseptic sterile (S-rp), with S. cerevisiae (H-rp) and with L. pontis (P-rp). Sample values are calculated based on the OD measurement at 590 nm and 100 % is defined as growth on 0,5 % yeast extract. Values are means of 3 experiments.

All fractions rp 0 representing the void volume of separation did not enable growth of *L. sanfranciscensis* therefore data are not shown. Some of the other fractions were able to promote growth under nitrogen limited conditions. The best results were obtained with fraction H-rp 2 (6 to 11.5 minutes) derived from yeast dough. These sample enabled 9 % growth compared to optimal growth conditions. On fractions H-rp 1, H-rp 3 and H-rp 4 2 % growth was detectable. Fractions P-rp2 and P-rp3, both resulting from a dough fermented with *L. pontis*, enabled a growth of about 4 %. Fractions P-rp1 and P-rp 4 did not promote any growth. Only one fraction from the aseptic acidified dough promoted growth, with S-rp 2 6 % were reached, all other fraction showed no significant growth. Hydrophilic proteins showed a better enhancement of growth than hydrophobic proteins.

In summarize it can be stated, that the ability to promote growth of *L sanfranciscensis* shows significant differences between the fractions. The activation of the samples varied between 0 and 30 %.

3.4 Depolymerization and proteolytic breakdown during sourdough fermentation

It was the aim of this study to analyze qualitative and quantitative changes of the GMP during sourdough fermentation. Two strains of lactobacilli were employed, *L. sanfranciscensis* LTH2581 and

L. pontis TMW1.397, representing the microflora of traditionally prepared sourdoughs (Vogel et al., 1999). To differentiate between effects of mere acidification, and specific metabolic activities of the lactic acid bacteria, sourdoughs were compared to acidified (pH 3.6) and neutral (pH 6.3) control doughs in which microbial metabolism was inhibited.

3.4.1 Microbial growth and proteolysis during dough fermentation.

Sourdough fermentations were carried out using two strains of lactobacilli and control fermentations were carried out aseptically at neutral and acid conditions to account for proteolytic events and modifications of the glutenin macropolymer in the absence of microbial metabolic activity. Additionally, sourdough fermentations were carried out in the presence of phosphate buffer to further differentiate between effects of microbial metabolism and a decreased pH. Cell counts, pH values, and the concentration of amino nitrogen for the various doughs are shown in Table 3.3.

Table 3.3 Cell counts, pH, and amino nitrogen contents of control doughs and sourdoughs.

fermentation time	neutral	acid	LTH2581	TMW 1.397	LTH2581, buffer
0h					
cell count	-		2×10^6	6×10^6	2×10^6
pH	6.21	3.56	6.25	6.18	6.51
amino nitrogen	4.0 ± 0.3	3.6 ± 0.5	4.1 ± 0.5	4.1 ± 0 3	4.3 ± 0.3
6h					
cell count [b)]	-	-	4×10^7	1×10^8	2×10^7
pH	6.23	3.69	5.28	4.52	6.03
amino nitrogen [c)]	5.5 ± 0.2	5.2 ± 0	5.9 ± 0.6	5.0 ± 0.2	5.5 ± 0.2
24h					
cell count	-	-	1×10^9	7×10^8	1×10^9
pH	6.51	3.67	3.62	3.60	3.76
amino nitrogen	8.8 ± 0.8	7.9 ± 0.6	7.4 ± 0.2	9.1 ± 0.2	6.6 ± 0 3

[a)] Cell counts of control neutral and acid doughs were less than 10^4 cfu g^{-1} throughout fermentation

[b)] Cell counts is expressed in cfu g $dough^{-1}$

[c)] Amino nitrogen is expressed in mmol glycine / kg dough

L. sanfranciscensis and *L. pontis* grew to cell counts of 10^9 cfu / g as typically observed in sourdough fermentations. The cell counts of control doughs were less than 10^4 cfu / g throughout fermentation, excluding a contribution of microbial metabolism to any of the analytical parameters observed. Samples were taken at the following times: unfermented dough (0h), 6 h of fermentation corresponding to a pH of 4.5 to 5.5 and exponentially growing cells in sourdoughs, and 24h of fermentation corresponding to a pH of 3.6 to 3.8. Growth and metabolism of lactobacilli ceased when the dough pH was reduced to 3.6 to 3.7, in agreement with the observation that growth of lactobacilli in sourdough is limited by dough pH (Gänzle et al., 1998). Addition of phosphate buffer to doughs fermented with *L. sanfranciscensis* LTH2581 delayed acidification without interfering with microbial growth or metabolism.

Overall proteolysis in doughs was quantified by determination of amino nitrogen. The concentration of amino nitrogen increased during fermentation and roughly doubled within 24h. No appreciable differences were observed between sourdoughs and neutral and acid control doughs with the exception of a noticeable lower proteolytic activity in lactic fermented doughs with addition of phosphate buffer. These data are in overall accordance with previous investigations using the same strains but a different wheat flour (Thiele et al., 2002).

3.4.2 Quantification and size distribution of SDS-soluble proteins.

SDS-soluble proteins were extracted from control doughs and sourdoughs and the relative changes in protein concentrations, and the size distribution of SDS-soluble proteins was determined by SEC. The fractionation range of SEC columns employed furthermore allowed to estimate the amount and size distribution of peptides resulting from proteolytic degradation of proteins. The quantification of proteins in SDS-extracts was performed using the 280 nm trace rather than the 210 nm trace to avoid interference with lactic acid and other non-protein carboxyl compounds co-eluting with amino acids or low molecular weight peptides. Examples of chromatograms of SDS-extracts are shown in Figure 3.14.

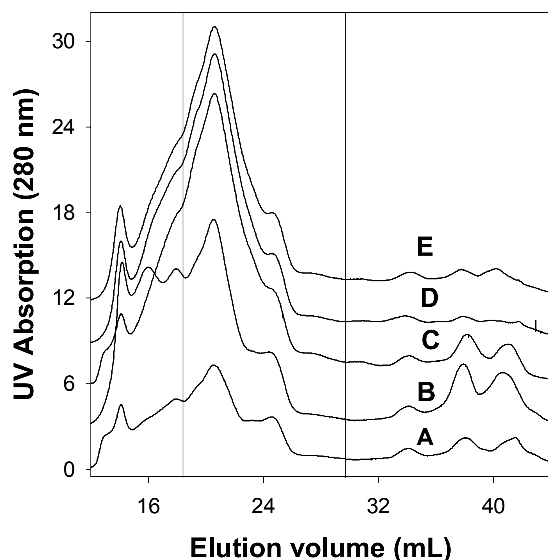

Figure 3.14. Separation of SDS soluble proteins from control doughs and sourdoughs by SEC. UV-280 nm traces represent extractions from neutral control dough at 0h (trace A), and extractions after 24h of fermentation from neutral control dough (trace B), acid control dough (trace C), sourdough with *L. sanfranciscensis* LTH2581 (trace D) and sourdough with *L. pontis* TMW 1.397 (trace E). fermented doughs after 24h as indicated. Chromatograms were offset by 3 AU. 12 – 18ml elution volume: polymeric proteins with MW greater 200 k, 18 – 30 ml elution volume: monomeric proteins, 30 – 44 ml elution volume: peptides and amino acids with MW less than 10 k.

In neutral control doughs was observed an increased solubility of proteins and the ratio of polymeric to monomeric proteins remained constant throughout fermentation. In contrast, proteins extracted from acid control doughs after 24h occurred mainly as monomers. Extracts from sourdoughs fermented with *L. sanfranciscensis* and *L. pontis* for 24h were highly similar to acid control doughs with respect to amount and size distribution of SDS-soluble proteins. However, as opposed to neutral and control doughs, the peak area representing low molecular weight peptides (MW about 500 – 5000) was strongly reduced in sourdoughs.

To quantify the relative protein contents SDS-soluble proteins in various doughs, and to determine the ratio of monomeric to polymeric protein, the peak areas corresponding to polymeric, monomeric proteins, and LMW peptides and amino acids were determined (Fig. 3.15).

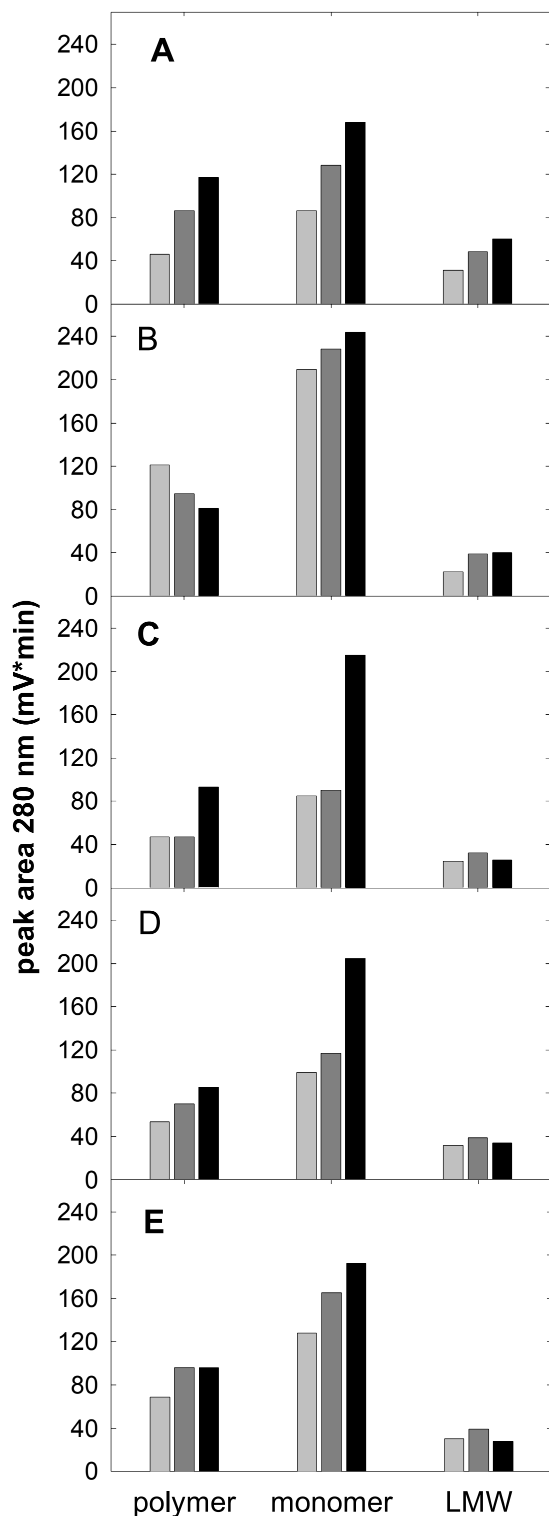

Figure 3.15. Amounts of polymeric and monomeric proteins, and low molecular weight peptides extracted with SDS from control doughs and sourdoughs. The amounts of proteins are expressed as AU x ml (280 nm) / 2 mg flour. Bars indicate extractions at 0h (■), extractions after 6 h (■), and extractions after 24h (■).

Panel A: neutral control dough,

panel B: acid control doughs,

panel C: sourdough fermented with *L. sanfranciscensis* LTH2581,

panel D: sourdough fermented with *L. pontis* TMW1.397,

panel E: phosphate buffered dough fermented with *L. sanfranciscensis* LTH2581.

In neutral control doughs, an increase was observed both of polymeric and monomeric proteins during fermentation. During acid control fermentation, an increased amount of SDS-soluble proteins was apparent already in unfermented dough, and during fermentation the amount of polymers decreased and the amount of monomers increased. The amounts of SDS-soluble proteins in sourdoughs was

comparable to neutral doughs after 0h of fermentation and comparable to acid doughs after 24h of fermentation. A decrease of peptides was observed in sourdoughs whereas an increase was apparent in both control doughs. The addition of buffer to doughs fermented with *L. sanfranciscensis* increased the solubility of proteins at 0 and 6 h of fermentation (Fig. 3.15). After 24h, no difference was observed between doughs fermented with *L. sanfranciscensis* in the presence or absence of phosphate buffer. Both the increase of SDS-soluble protein and the shift of peak areas from polymeric to monomeric proteins indicate a depolymerization of gluten proteins during fermentation at acidic conditions induced by addition of lactic and acetic acids (acid control doughs), or by sourdough fermentation.

To verify that an increased protein content in SDS-extracts corresponds to a decreased content in dough of SDS insoluble GMP, the residue of the SDS-extraction was extracted with SDS-DTT. Examples of SEC-separations of SDS-DTT extracts are shown in Fig. 3.16.

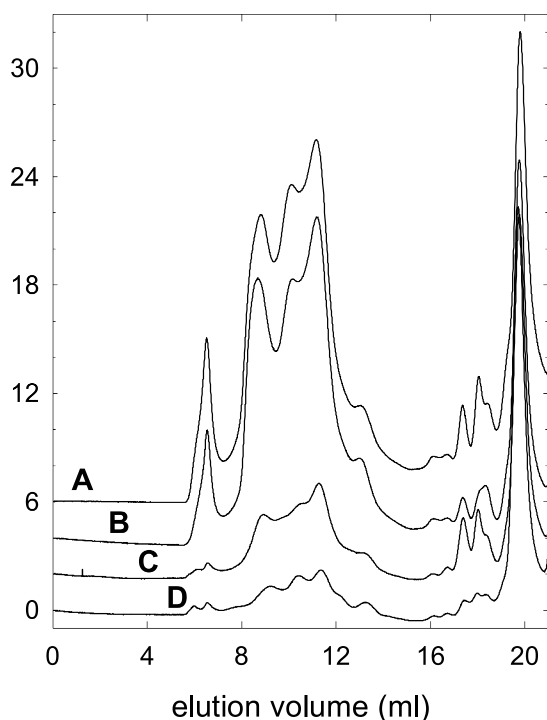

Figure 3.16. Separation of SDS insoluble, SDS-DTT soluble proteins from neutral control dough and sourdough by SEC. UV-280 nm traces represent extractions from neutral control dough at 0h (trace A), unfermented sourdough (t 0h) with *L. sanfranciscensis* LTH2581 (trace B), and extractions after 24h of fermentation from neutral control dough (trace C), and after 24 h fermentation with *L. sanfranciscensis* LTH2581 (trace D). Chromatograms were offset by 2 AU.

An increased values for SDS-soluble protein corresponded to decreased SDS-insoluble, SDS-DTT soluble proteins in all doughs at any time (fig 3.15, data not shown).

To gain insight in the composition of proteins solubilized by sourdough fermentation, SDS-soluble proteins from doughs were analyzed by SDS-PAGE (Figure 3.17).

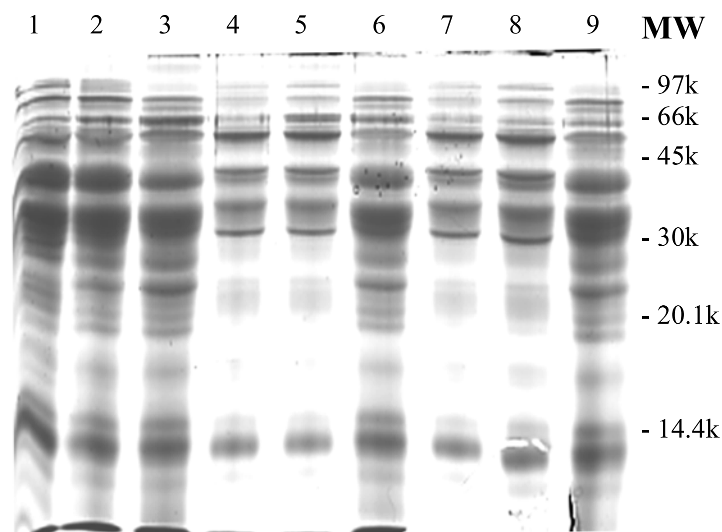

Figure 3.17. Separation of SDS-soluble proteins from control doughs and sourdoughs by SDS-PAGE. Samples were applied to represent equal amounts of flour. Lanes 1, 2 and 3, acid control doughs extracted after 0 h, 6 h and 24 h, lanes 4, 5, and 6, sourdough with *L. sanfranciscensis* LTH2581 extracted after 0 h, 6 h and 24 h, lanes 7, 8, and 9: sourdough with *L. pontis* TMW 1.397 dough extracted after 0 h, 6 h and 24 h. The migration of the molecular weight markers in the gel is indicated.

In neutral control doughs, the qualitative composition of SDS-soluble proteins was comparable to that of salt-soluble proteins throughout fermentation (data not shown) and only small amounts of glutenin and gliadin were detectable. In acid control doughs, glutenins and gliadins were SDS-soluble, indicating that acidity inhibited the formation of the GMP. After 24h of acid control fermentation, the band intensity of individual high molecular weight (HMW) glutenins decreased and additional bands were detectable with a molecular weight of about 30 k. The composition of SDS-soluble proteins extracted from sourdoughs at time 0h was comparable to neutral control doughs and after 24h, the SDS-PAGE profile of sourdoughs was comparable to that of acid control doughs. Taken together, the qualitative and quantitative analyses of SDS-soluble protein suggest partial hydrolysis and depolymerization of the GMP during fermentation at acidic conditions.

3.4.3 Sequential extraction of gluten proteins and qualitative and quantitative analysis by SDS-PAGE and RP-HPLC.

In order to enable analysis of proteins remaining in the GMP after fermentation, proteins from various dough samples were sequentially fractionated in water / salt soluble, propanol soluble, and propanol-

DTT soluble proteins and separated electrophoretically. Differences between doughs in either propanol-soluble or propanol-DTT soluble proteins were not detected in unfermented doughs (data not shown). The analysis by SDS-PAGE of propanol soluble proteins extracted after 24 h of fermentation is shown in Figure 3.18A, and propanol-DTT soluble proteins after 6 and 24 h of fermentation are shown in Figures 3.18B and 3.18C, respectively.

Protein patterns of propanol soluble proteins extracted from neutral control doughs did not show appreciable differences over fermentation time. Extracts from acid control doughs and sourdoughs were characterized by the presence of HMW glutenin subunits after 6 and 24h of fermentation, and proteins with a MW of $2 – 3 \times 10^4$ that were not present in unfermented doughs or in neutral control dough. Changes in propanol-DTT soluble proteins extracted from neutral control doughs were not observed and virtually identical protein patterns were detected in all unfermented doughs (Figures 3.18B and 3.18C, data not shown).

After 6 h of acid aseptic incubation, degradation of individual HMW subunits and an additional protein with a MW of about 6×10^4 as well as proteins in the MW range of $2 – 3 \times 10^4$ were detected. The protein patterns of sourdoughs were comparable to that of neutral control doughs after 6h of fermentation. After 24 h of fermentation, both acid control doughs and sourdoughs were characterized by virtually quantitative degradation of individual HMW glutenin subunits and the presence of hydrolysis products with a MW of 6 and 2.5×10^4

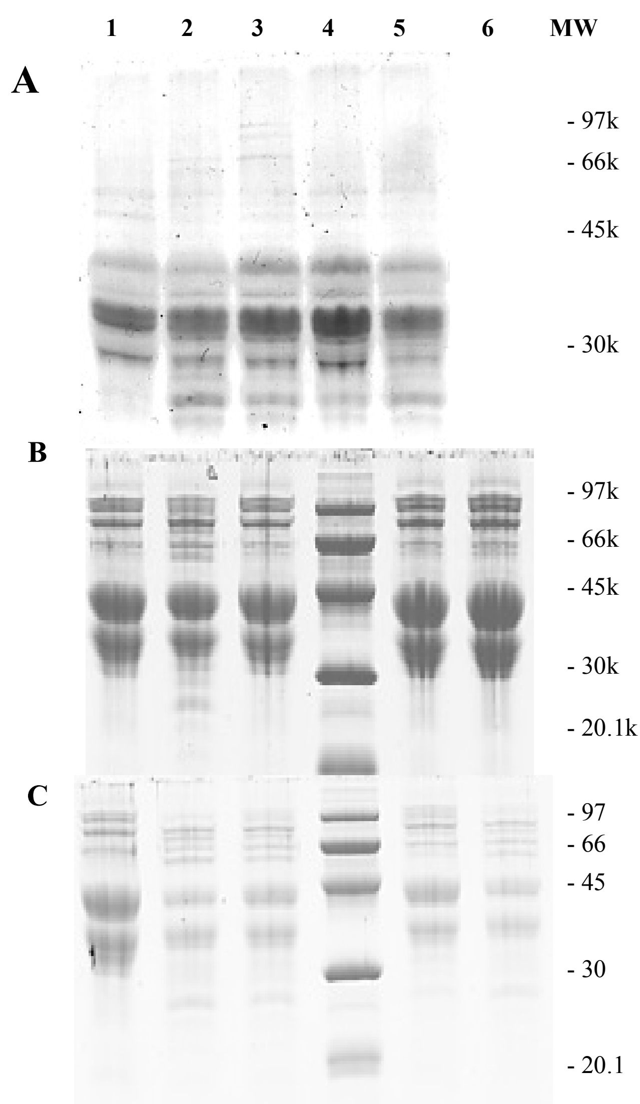

Figure 3.18. SDS-PAGE analysis of propanol and propanol-DTT soluble proteins extracted from control doughs and sourdoughs. Samples were applied on the gel to represent equal amounts of flour. Panel A. propanol – water soluble proteins extracted after 24 h. Lane 1: neutral control dough, 2: acid control dough, 3: sourdough (*L. sanfranciscensis* LTH2581) 4: buffered sourdough (*L. sanfranciscensis* LTH2581), 5: sourdough (*L. pontis* TMW1.397). Panel B: Propanol – acetic acid – DTT soluble proteins extracted after 6 h. Lane 1: neutral control dough, 2: acid control dough, 3: sourdough (*L. sanfranciscensis* LTH2581), 4: MW marker, 5: buffered sourdough (*L. sanfranciscensis* LTH2581), 6: sourdough (*L. pontis* TMW1.397). Panel C: Propanol – acetic acid – DTT soluble proteins extracted after 24 h, lanes as in panel B.

Quantification of the amounts of protein extracted with propanol and propanol-DTT was performed by separation of proteins by RP-HPLC according to Wieser et al. (1998) and determination of relative amounts of protein through integration of the 210 nm trace. Chromatograms obtained from neutral unfermented dough, and 24h fermented dough at neutral aseptic, acid aseptic conditions, and sourdough fermented with *L. sanfranciscensis* LTH2581 are shown Figure 3.19, and the peak areas correlating to total protein content of the extracts are shown in Figure 3.20.

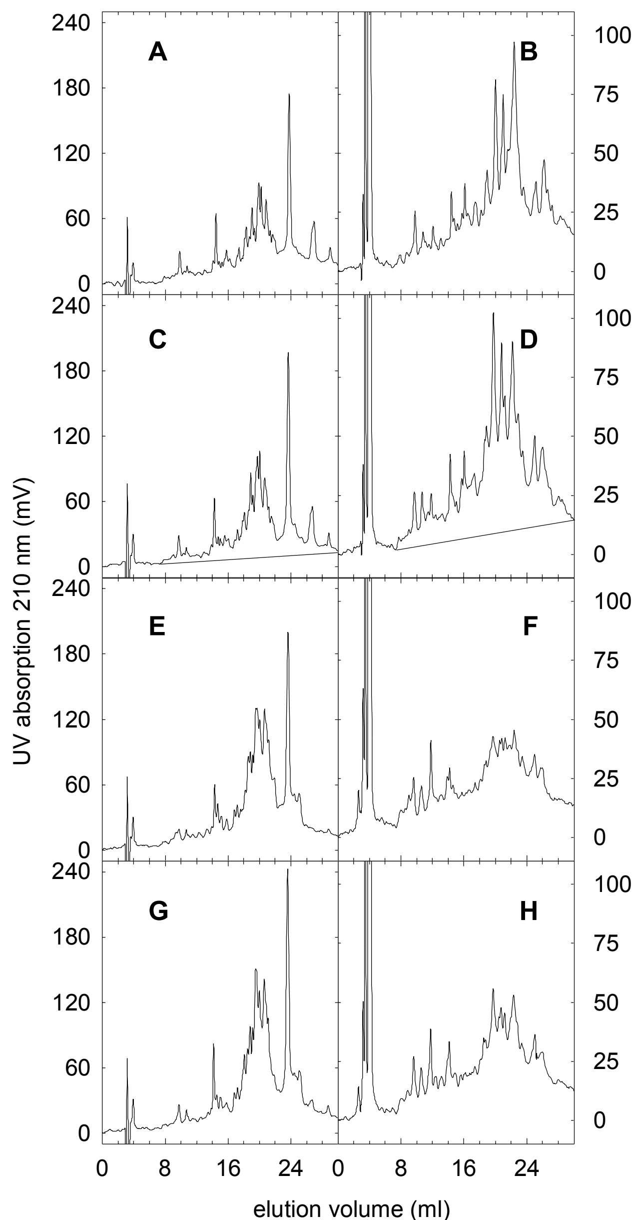

Figure 3.19 Separation of propanol soluble proteins and propanol-DTT-soluble proteins extracted from control doughs and sourdoughs by RP-HPLC. Left panels (A, C, E, and G): propanol soluble proteins. Right panels (B, D, F, and H): Propanol-DTT-soluble proteins. Shown are chromatograms of extracts of unfermented neutral dough (A, B), neutral control, 24h fermented dough (C, D), acid control, 24h fermented dough (E, F), and sourdoughs, 24 h fermented with *L. sanfranciscensis* LTH2581 (G, H). The amount of protein applied to the column represented 3 mg of flour.

The peaks were not assigned to individual gluten subunits because, after fermentation at low pH, most glutenin subunits were present in the propanol extract and new peaks were observed in propanol-DTT extracts upon fermentation mainly in the hydrophilic part of the gradient (6 – 16 min), which presumably arise from partial hydrolysis of glutenin subunits also observed by SDS-PAGE.

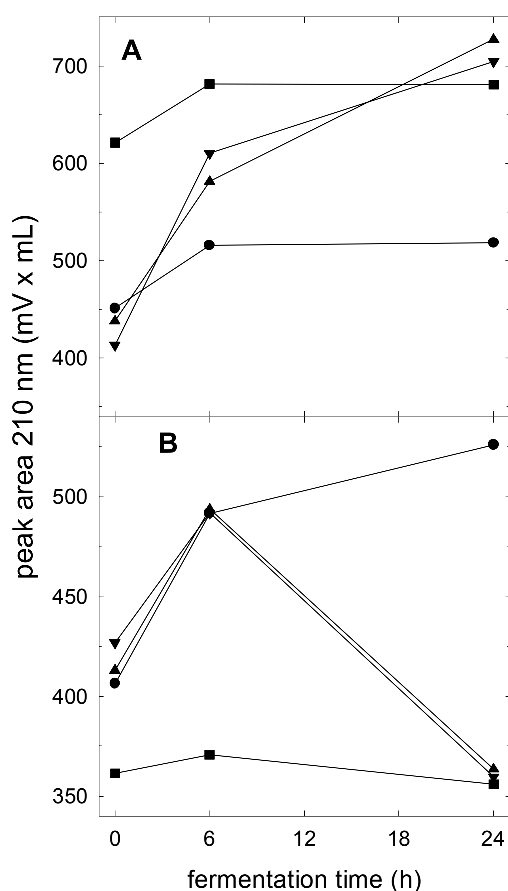

Figure 3.20. Quantification with reversed-phase HPLC of proteins extracted from control doughs and sourdoughs. The amounts of proteins are expressed as AU x ml (210 nm) / 3 mg flour. Shown are propanol - water soluble proteins (panel A) and propanol – acetic acid – DTT soluble proteins (panel B). Symbols indicate extracts from neutral control (●) and acid control doughs (■), and sourdoughs fermented with *L. sanfranciscensis* LTH2581 (▲), or *L. pontis* TMW1.397 (▼). Experimental error is smaller than the symbol size.

In neutral control doughs, a slight increase in protein concentration was observed in propanol extracts over time whereas an strong increase was observed in propanol-DTT extracts. In contrast, a strong decrease of propanol-DTT soluble proteins was observed after 24 h of fermentation of acid control doughs and *L. sanfranciscensis* and *L. pontis* doughs. Correspondingly, a strong increase was detected in propanol soluble proteins, indicating decreased intermolecular disulfide bonding and glutenin depolymerization. The sum of proteins in either extract increased in all doughs during fermentation by about 20 % (neutral control, *L. sanfranciscensis* and *L. pontis* doughs) and 10% (acid control), indicating the proteolytic degradation of gluten proteins to water soluble amino acids or peptides did not play a major role.

3.5 Depolymerization and proteolytic breakdown of FITC-Proteins

3.5.1 Sequential extraction, labeling and purification of wheat proteins.

Sequential extraction was carried out to obtain albumin/globulin, gliadin, and monomeric glutenin fractions from wheat flour. Polymeric glutenin was obtained with a solvent containing 1.5% SDS. After dialysis of labeled proteins, fluorescence was mainly attributable to unbound FITC (data not shown). Unbound dye was quantitatively removed from fluorescent proteins by preparative SEC using G 10 Sephadex. The fractions containing purified proteins were dried and analyzed by SDS-PAGE, and by size exclusion chromatography. The fluorescence pattern of the labeled and purified protein fractions after separation by SDS-PAGE is shown in figure 3.21.

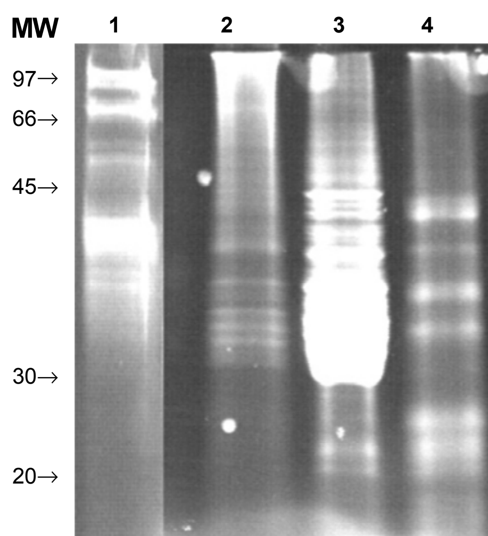

Figure 3.21. Separation of FITC labeled wheat protein fractions by SDS-Page and detection of fluorescence. Lane 1 reduced sample of SDS-extracted glutenin after dialysis. Lanes 2, 3, 4 and 5 non-reduced samples of SDS-extracted glutenin, gliadin and albumin/globulin, respectively, after gel chromatography. The migration of the molecular weight markers in the gel is indicated for the reduced and the non reduced SDS-Page.

The proteins of all fractions were labeled with FITC. The monomeric glutenin fraction (extraction with solvent 3A containing DTT) was completely unlabeled and only very small amounts of unbound dye were visible (data not shown).

Comparison of the protein bands obtained by fluorescence measurements and Coomassie staining showed virtually identical band patterns. The albumin and globulin fraction contained a wide range of labeled peptides with an MW of less than 14 000 in addition to proteins. Protein patterns of gliadin and glutenin fractions as visualized by Coomassie-staining were not modified by the labeling procedure (data not shown) and no proteins with an relative molecular weight (MW) of less than 24 000 were observed in the labeled gliadin and glutenin fractions (Figure 3.21), indicating that

proteolytic degradation through gluten associated proteases did not occur during the labeling procedure. The molecular size was not significantly modified by the labeling. Upon analysis of the polymeric glutenin fraction by SDS-PAGE without reduction of the sample, mainly protein complexes with an MW greater than 94 000 and only small amounts of monomeric LMW glutenin subunits were found. Separation of the same fraction by SDS-PAGE with reduction of the sample (figure 3.21, lane 1) demonstrated that HMW as well as LMW glutenin proteins were equally labeled.

In all labeled protein fractions, the ratio of bound and unbound fluorescence was quantified by SEC. The results are for FITC-gliadin are shown in figure 3.22.

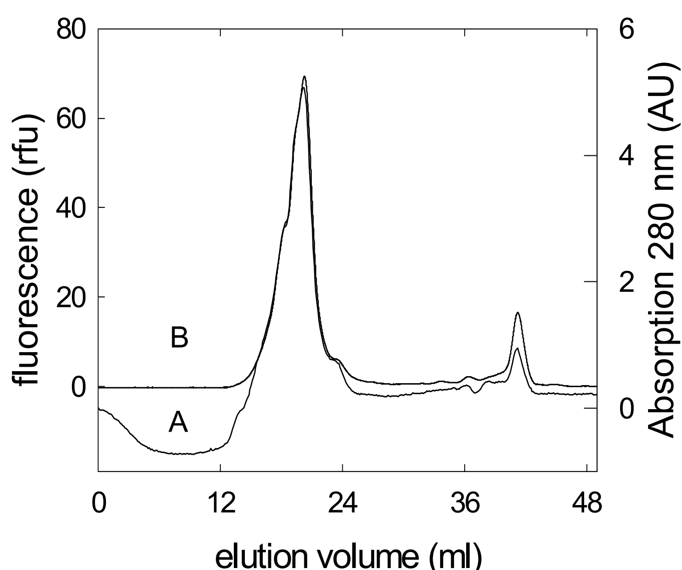

Figure 3.22. Separation of FITC-labeled gliadins after gel chromatography by SEC, fluorescence is compared to adsorption at 280 nm. Trace A: UV-280 traces specifically represent the protein content. Trace B: Fluorescence was excitated at 485 nm and emissioned at 520 nm.

Fluorescence as well as UV absorption at 280 nm were used for labeling control. Both detection methods show comparable chromatograms. Most UV absorption and 91% of fluorescence is found between 15 mL and 24 mL elution volume, corresponding to proteins. A peak containing 9% of total fluorescence occurred after 40 mL, corresponding to compounds with a relative molecular weight (MW) of less than 500. A comparable result was obtained upon analysis of the labeled albumin /

globulin fraction. Fluorescence of low molecular weight compounds (MW < 500) amounted to 30% in the glutenin fraction.

3.5.2 Correlation of protein size and elution volume of SEC

In addition to the calibration of the SEC columns with external standards, the SEC-columns were calibrated using SDS-soluble wheat proteins. Neutral dough was fermented aseptically for 24h, SDS-soluble proteins were extracted and analyzed by SEC (figure 3.23)

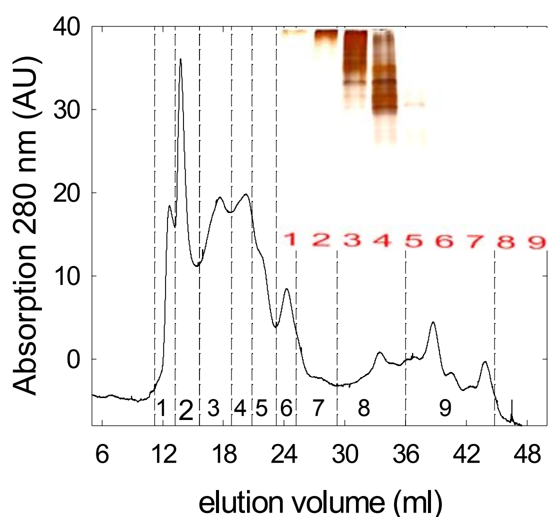

Figure 3.23: Fractionation of SDS soluble proteins by SEC and separation of these fractions by SDS-Page without reduction. UV-280 nm trace represent extraction from a aseptic neutral control dough fermented for 24 hours. Different fractions are indicated with dotted lines. Equal amounts of fractions were added on SDS-Page and silver stained. 12-18 ml elution volume: polymeric proteins with MW greater 200k, 18-24 ml elution volume: monomeric proteins, 24-44 ml elution volume: peptides and amino acids.

To determine the elution volumes representing polymeric and monomeric proteins as well as peptides and amino acids, the eluate was fractionated and analyzed by SDS-PAGE without reduction of the samples. The electrophoretic separation of the unreduced fractions is shown in the inlay of figure 3.23. Lanes 1, 2 and 3 contain polymeric proteins. In lane 3 to lane 5, monomeric proteins and peptides with MW larger than 5000 were detected. All other lanes do not contain any proteins stained by silver and were assumed to be peptides or amino acids. Results demonstrate that polymeric proteins elute at 12 to 18 mL, monomeric proteins at 18 to 24 mL and amino acids and peptides with a molecular weight of less than 5000 elute at 30 to 44 mL.

3.5.3 Influence of modified proteins and protease on dough fermentation

To determine possible influences of FITC proteins on microbial growth caused by the addition of labeled proteins or exogenic protease, all FITC-fractions and the fungal protease were tested in dough

fermentations inoculated with *L. pontis* as described in Table 2.7, and cell counts were determined after 24 hours. Lactobacilli grew to cell counts typically observed in sourdough fermentations, i.e. 10^9 cfu / g, in those doughs containing protease, albumin/globulin, or gliadin. When FITC-glutenin was added, cell counts were significantly lower and only 5 x 10^7 cfu /g dough. Nevertheless, the acidification activity of the microorganisms the same as that observed in other doughs. The absence of contaminants (less than 1 % of the total cell count) in sourdough fermentations was verified by determination of the colony morphology.

Sourdough samples were extracted immediately after dough mixing and after 24 hours and were subsequently analyzed by SEC. All doughs showed virtually identical UV- adsorption chromatograms (data not shown), the differences in the overall amount of proteins and their size distribution was within experimental error (5%). Therefore the amount of added proteins can be neglected compared to the normal protein content of a wheat dough and the amount of glutenin proteins added did not affect the properties of the protein gel.

3.5.4 Proteolytic degradation of FITC-proteins

Degradation of proteins in fermented wheat dough is catalyzed either by microbial proteases or by endogenic wheat proteases and peptidases. To determine the effect of wheat and microbial proteases on individual substrates, FITC-labeled albumin/globulin, gliadin and glutenin fractions were separately added to neutral and acid control doughs, and sourdoughs fermented *L. pontis* and *L. sanfranciscensis*. Additionally, fermentations with fungal protease with and without *L. pontis* were carried out (Table 2.7). Samples were analyzed with respect to the size distribution of proteins by SEC and fluorescence detection. The percentage of total fluorescence recovered from dough samples as proteins (from 12 to 24 ml) and peptides/amino acids (from 24 to 40 ml) is shown in table 3.4.

Table 3.4: Recovery of FITC-gliadin and FITC- glutenin as proteins and hydrolysis products from doughs fermented for 24 hours. Values are expressed as percentage of the fluorescence chromatogram areas corresponding to proteins and peptides relative to the total fluorescence chromatogram areas of unfermented doughs.

Dough	**FITC gliadin**		**FITC glutenin**	
	proteins[a)]	peptides	proteins	peptides
neutral control 0 h	75[b)]	25	61	39
neutral control	65	28	45	34
acid control	71	34	50	50
L. pontis	62	32	52	51
L. sanfranciscensis	63	36	45	53
acid control, protease	37	57	32	67
L. pontis, protease	10	90	12	88

[a)] The peak area from 12 ml to 24 ml elution volume corresponded to proteins, the area from 24 to 40 ml corresponded to peptides and amino acids.

[b)] Percentage values were calculated based on the total fluorescence of gliadins or glutenins after 0 hours fermentation time

The percentage values were calculated based on the total fluorescence of each dough at time zero and 99 ± 3.5 of total fluorescence were recovered after fermentation. Due to incorporation of fluorescent glutenin into the SDS-insoluble protein gel in neutral doughs (see below), the sum of fluorescence (protein) and fluorescence (amino acids, peptides) was 79% only.

In neutral control doughs no significant increase was detectable during fermentation in peptides/amino acids when either labeled gliadins or glutenins were added to the doughs. The acid control dough as well as the sourdoughs showed an increase of chromatogram area corresponding to peptides and amino acids from 39% to about 50% for glutenins and from 25% to 33% for gliadins (table 3.4, Figure 3.24A).

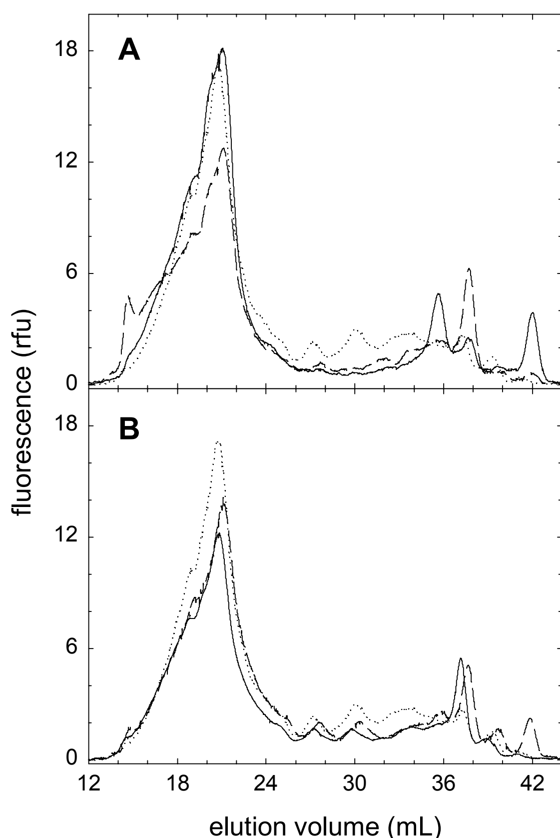

Figure 3.24: Separation of SDS soluble proteins from doughs containing fluorescence labeled gliadin by SEC. Panel A. Fluorescence trace represent extractions from neutral control dough at 0 hours (solid line), and extractions after 24 hours of fermentation from neutral control dough (dashed line), and acid control dough (dotted line). Panel B. Fluorescence trace represent extractions after 24h of fermentation from sourdough with *L. pontis* (solid line), sourdough with *L. sanfranciscensis* (dashed line); the extraction from 24 h fermented acid control dough is furthermore included for comparison (dotted line).

No significant differences were observed between sourdoughs and acid control doughs when the sum of low molecular weight fluorescence were compared. However, differences in size distribution of fluorescence labeled peptides in acid control doughs and sourdoughs were observed (figure 3.24B). Between 28 mL and 35 mL elution volume, the amount of fluorescence was higher in acid control doughs compared to sourdoughs. Between 35 and 40 mL elution volume corresponding to small peptides or amino acids, the highest fluorescence was found in doughs fermented with lactobacilli.

It was evaluated whether full proteolytic degradation of FITC gliadin and FITC-glutenin is achieved by addition of a commercially available fungal protease. Acid control dough and sourdough containing *L. pontis* were fermented for 24 hours. Based on the fluorescence chromatograms, the percentage of proteins and peptides/amino acids was calculated (Table 3.4). In aseptic, acidified doughs in the presence of protease, relative amounts of peptides increased during fermentation from 26 % to 57 % for gliadin and from 39 to 67% for glutenin. In both fermentations less than 50 % of the labeled proteins were degraded. To rule out influences of the FITC-labeling on the enzyme specificity, this

result was compared with the size distribution of all proteins available in dough fermentation (210 nm trace), which showed similar results (data not shown), indicating that unlabeled proteins were also not completely degraded. When *L. pontis* was added to the fermentation, the content in FITC labeled peptides increased in both fermentations to about 90%. This result was also verified for all proteins with the absorption at 210 nm. Dough containing both lactobacilli and protease showed a significantly higher proteolytic activity, indicating synergistic activity of the exogenic protease and the proteolytic system of *L. pontis*.

3.5.5 Incorporation of proteins into the gluten macropolymer

SEC analysis of SDS-soluble proteins allow a reasonable estimation of the incorporation of the FITC labeled proteins into the SDS-insoluble gluten macropolymer. During dough mixing, the gluten macropolymer partially depolymerizes and in the subsequent resting time the disulfide bonds become restored and the amount of protein gel increases (Skerritt et al., 1999, Weegels et al., 1996 and 1997). This biochemical process is dependent on enzyme catalyzed processes in wheat dough. FITC-glutenin was added to all doughs (Table 2.7) to determine whether the modified proteins are integrated into the gluten macropolymer. The fluorescence trace of samples from neutral control dough after 0, 7, and 24 h fermentation is shown in figure 3.25A.

At time zero, 20 % of the fluorescence is found in polymeric proteins and 41 % in monomeric proteins, corresponding to a ratio of polymeric to monomeric proteins of 0.49. After 24 hours, less fluorescence is SDS-extractable, the overall fluorescence recovered from the aseptic neutral dough decreased to 79 % compared to the fluorescence recovered from unfermented dough. There was virtually no increase of fluorescent peptides or amino acids, but a high molecular weight peak (MW > 2×10^6) appeared after 14 ml elution volume and the ratio of polymeric to monomeric proteins increased to 0.65. These results indicate that under neutral conditions fluorescence labeled glutenins polymerize and are partially incorporated in the SDS-insoluble GMP.

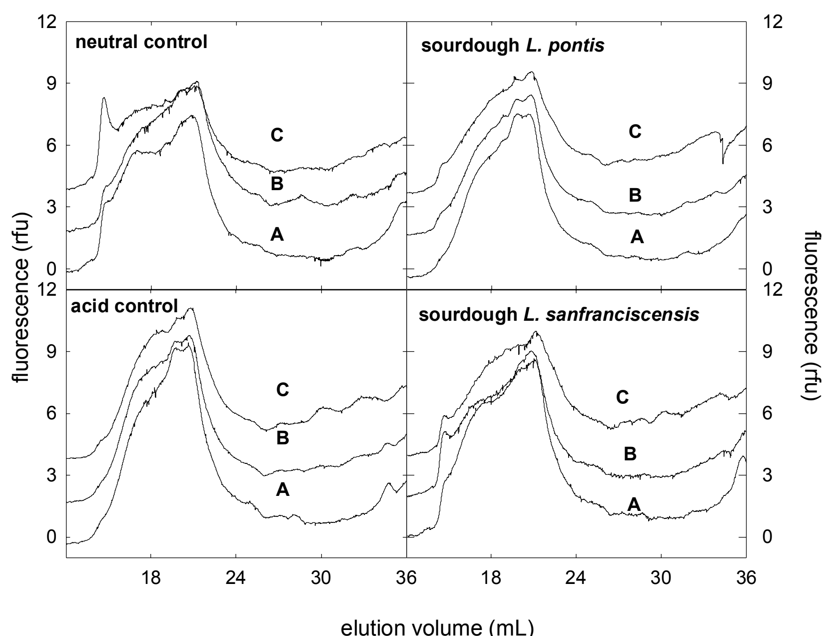

Figure 3.25: Separation of SDS soluble proteins from doughs containing fluorescence labeled glutenin by SEC. Fluorescence trace represent extractions from doughs extracted after 0 (A), 7 (B) and 24 (C) hours. Shown are chromatograms for neutral control doughs, acid control dough, sourdoughs fermented with *L. pontis*, and sourdoughs fermented with *L. sanfranciscensis*. Polymeric proteins elute at 12 to 18 mL, monomeric proteins at 18 to 24 mL and peptides/amino acids from 24 to 44 ml. Chromatograms were cut-off at 36 min to highlight shifts in HMW-peaks.

Fluorescence chromatograms of SDS-soluble proteins extracted from an acid control dough with addition of FITC-glutenin are shown in figure 3.25B. The overall fluorescence did not change significantly during fermentation. As opposed to neutral controls, less than 15 % of the fluorescence in acidified samples is found in the polymeric protein fraction. A incorporation into polymeric SDS-soluble proteins or the SDS-insoluble glutenin macropolymer was not observed, indicating that polymerization of glutenin subunits does not occur at low pH.

The influence of microbial metabolism on formation of the protein gel was determined by SEC analysis of SDS-soluble proteins from sourdoughs fermented with *L. pontis* and *L. sanfranciscensis*. The chromatograms from doughs with incorporated FITC-glutenin are shown in figure 3.25 for *L. pontis* and *L. sanfranciscensis*, respectively, after 0, 7, and 24h fermentation. Doughs fermented with *L. pontis* and *L. sanfranciscensis* showed virtually identical chromatograms compared to neutral doughs during the first 7 hours. The overall fluorescence decreased in both doughs, and the peak corresponding to polymeric proteins appeared. After acidification of the doughs due to microbial

metabolism, the polymerization stopped, the overall amount of fluorescence increased again to levels of unfermented doughs.

Incorporation of FITC-gliadin in polymeric gluten was determined during fermentation in aseptic neutral and acidified doughs, and in sourdoughs. To determine polymerization over time, the fluorescence chromatograms of extracts from doughs were integrated and the ratio between polymeric and monomeric proteins was calculated. The results are shown in table 3.5.

Table 3.5: Recovery of FITC-gliadins as polymeric and monomeric proteins from neutral and acid control doughs and sourdoughs fermented with *L. pontis* and *L sanfranciscensis*. Shown are the ratios of peak areas corresponding to polymeric and monomeric fluorescent proteins.

Dough	**Ratio polymeric/monomeric proteins**[a)]		
	0 hours	7 hours	24 hours
neutral control	0.23 [b)]	0.37	0.41
acid control	0.18	0.18	0.20
L. pontis	0.21 [b)]	0.30	0.25
L. sanfranciscensis	0.23 [b)]	0.38	0.22

[a)] Values were calculated based on the fluorescent trace of SEC-analysis. Elution volumes of 12 to 18 mL corresponded to polymeric proteins, elution volumes from 18 to 24 mL corresponded to monomeric proteins.

[b)] These values were used to determine the experimental error range, the acid control dough showed already a modified dough rheology due to the acid addition.

The total fluorescence recovered from the doughs after fermentation, including the neutral dough, varied by less than 6 %, indicating that incorporation of FITC-gliadin in the SDS-insoluble GMP did not occur. In the neutral control dough, the ratio of polymeric to monomeric FITC-gliadin increased from 0.23 to 0.41 after 24h of fermentation (table 3.5, Figure 3.24A). In acid control doughs there is virtually no change during fermentation time, the ratio remained stable between 0.18 and 0.20. A polymerisation of FITC-gliadin at acidic conditions can therefore be ruled out. During the first 7 hours of fermentation with lactobacilli, corresponding to a dough pH > 4.5, the ratio increased from about 0.22 at 0h to 0.30 for *L. pontis* and to 0.38 for *L. sanfranciscensis*. After 24 hours and microbial acidification of the doughs to pH 3.6, the ratio decreased again to about 0.23 in extracts from either

sourdough. Taken together, the results indicate that polymerization and depolymerization of gliadin and glutenin are strongly affected by dough pH.

Incorporation into the GMP was also tested with FITC-albumin/globulin. After 24 hours of fermentation in any of the doughs, virtually no increase in low molecular weight fluorescence was found. Fluorescence is exclusively found in monomeric proteins and less than 2 % of FITC-albumins and globulins were recovered as polymeric proteins (data not shown).

3.6 Amino acid determination by IPAD

It was the aim of this study to establish a sample preparation for the fast and flexible separation of sugars and amino acids, including arginine, from food samples and the subsequent quantification of either group of compounds by anion exchange chromatography and integrated pulsed amperometric detection.

3.6.1 Absorption of amino acids and removal of sugars

Amphoteric ions like amino acids can be retarded either by cation or by anion exchange. For two reasons the cation exchange resin was preferred. (i) most ion exchange matrices are based on silica gel, which is not stable under alkaline conditions. (ii) Sample preparation often includes a protein removal step by adding perchloric acid or trichloroacetic acid and concomitant acidification to 2.0.

The first step of sample preparation was performed with sugar and amino acid standards according to Method 1 (Thurman and Mills, 1998) and Method 2 (Anonymous, 1998). With both methods all amino acids were completely bound to the matrix (data not shown). To remove all sugars from the void volume, the column is flushed with 500 µl of water or 10% methanol after complete permeation of the sample volume. The elution of sugars was more effective with the addition of 10% methanol but methanol eluted at 3 minutes retention time and was detected by IPAD.

In order to determine the effect of pH and methanol concentrations on the binding of amino acid to the resin, standards were diluted 1 to 10, and applied to extraction columns. The following conditions were evaluated: 0.01 N to 0.05 N HCl, 0% methanol, and 0.01 N HCl, 0 to 50% methanol. Except for the samples containing 0.05 N HCl, all amino acids were bound and all sugars were eluted.

To test the maximal amount of amino acids retained by the columns, an amino acid standard was adjusted to 0.01 N HCl and 10% methanol, applied to the column in 1 ml portions and the eluent was collected in 2 ml fractions. The fractions were analyzed by HPLC and the chromatograms are shown in Figure 3.26.

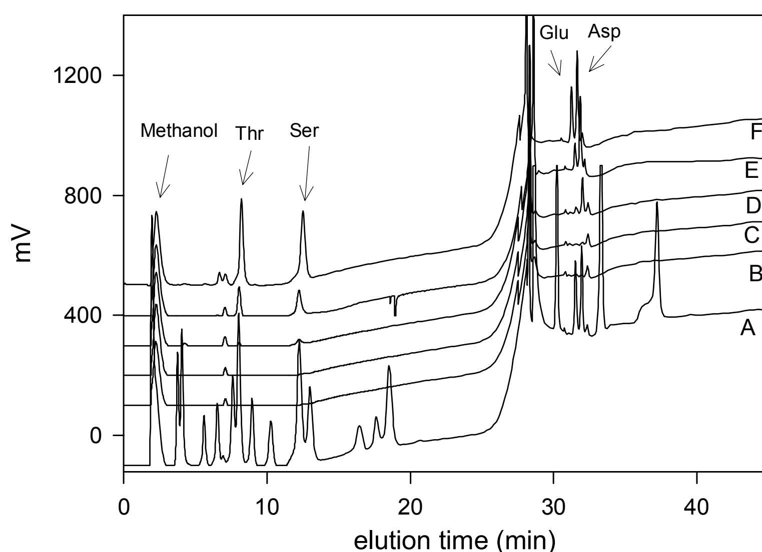

Figure 3.26: Capacity of ion exchange resin for retention of amino acids. Amino acid standard sample was successively applied in 1 ml fractions to a solid phase extraction column, each fraction containing 1.85 µmol amino acids. The eluent was collected in 2 ml portions and analyzed by HPLC-IPAD. Trace A: standard sample before application, traces B through F: eluate of first to fifth application of amino acid standard.

All samples were separated with a gradient based on the recommendation of the manufacturer, which was modified to improve the separation of samples containing high salt concentrations (table 1), and to improve the separation during the first 25 minutes. A cleaning step with high concentrations of sodium acetate and sodium hydroxide was performed prior to application of each sample to condition the column.

The first 2 fractions did not contain any amino acids. Aspartate and glutamate were the first amino acids that could be detected from the 3rd fraction on. These amino acids containing acidic side groups have the lowest isoelectric point (pH_i), which is for aspartate 2.77 (Jakubke and Jeschkeit, 1992). For a 90% protonation of aspartate, the sample pH calculated with the Henderson-Hasselbach equation must be below 1.8. Because of this weak protonation at pH 2 aspartate and glutamate eluted first. Based on

the concentrations of amino acids in the standard solution applied to the column, it was determined that the maximal amount of amino acids per SPE column avoiding loss of aspartate and glutamate was 3.7 µmol amino acids per 100 mg cationic exchange matrix.

3.6.2 Influence of salt and pH on the elution of amino acids

The second step of solid phase separation is the elution of bound molecules. Generally, desorption can be achieved by an increase of the pH, or an increased ionic strength of the solvent by addition of cations. The resin used in this work is not compatible with pH values required for desorption of arginine, histidine, lysine, and ornithine in their negatively charged forms. Elution by increased ionic strength is possible with strong acids or divalent cations. H^+ ions have a low relative selectivity of 9 and the divalent cations Mn^{2+} and Ca^{2+} have a relative selectivity of 28 and 46, respectively (Thurman and Mills, 1998). Mn^{2+} and Ca^{2+} will replace the amino acids more efficiently and can be utilized at a neutral pH.

To determine conditions allowing quantitative elution of amino acids, columns were loaded with 1 ml amino acid standard, washed with 1 ml water, and eluted with 3 ml 0.1 N HCl, (Thurman and Mills, 1998; Anonymous, 1998). The eluent was collected in 1 ml fractions and amino acid concentrations were determined by HPLC. Most amino acids were not completely eluted by hydrochloric acid and the amino acids lysine, histidine and phenylalanine could not be found in the hydrochloric acid eluate. Elution of amino acids with 0.1 N HCl is therefore not an appropriate method. In the next step, the sequential use of 0.1 N HCl, 0.1 N $CaCl_2$ and water was evaluated. Quantitative elution of phenylalanine, arginine, lysine, ornithine and histidine was not achieved with 0.1 N HCl followed by 0.1 N $CaCl_2$ or vice versa (data not shown).

Elution was performed with exclusive use of $CaCl_2$ solution with concentrations ranging from 0.025 M to 1.0 M CaCl2 and with elution volumes between 1 and 5 ml. Solutions with Ca^{2+} concentrations below 0.1 M did not elute histidine, and basic as well as aliphatic amino acids were not recovered quantitatively. If samples were eluted with solvents containing $CaCl_2$ concentrations 0.5 M or more, the high salt concentration interfered with the subsequent quantification of amino acids by HPLC.

Using $CaCl_2$ concentrations between 0.1 and 0.2 M, quantitative recoveries were found. The recoveries for all amino acids using a elution with 0.2 M $CaCl_2$, 1 ml H_2O ranged from 90% for arginine and 126% for cysteine (table 3.6).

This experiment was reproduced 4 times and the experimental error was generally 10 % or less. The experiment was repeated by application of different concentrations of amino acids (4.2 nmol, 21 nmol, 42 nmol, or 84 nmol) to cation exchange columns, followed by elution with 2 ml of 0.2 M $CaCl_2$ and 1 ml of H_2O. Samples were appropriately diluted and amino acid concentrations in the eluent determined by HPLC analysis. The concentration of amino acids determined by HPLC was plotted against the amount of amino acids applied to the cation exchange resin and the correlation coefficients are shown in table 3.6. The correlation coefficients were in the range of 0.84 and 0.99 for all amino acids employed. A value lower than 0.94 was only found for glutamine.

To verify that complete separation of amino acids and carbohydrates was achieved with this method, a mixture of the amino acid and carbohydrate stock solutions was applied to the cation exchange column and eluted with 0.2 M $CaCl_2$, 1 ml H_2O. The separated carbohydrate and amino acid fractions were analyzed by HPLC and the IPAD signal was integrated using the integration periods specific for carbohydrates as well as the integration period allowing detection of both amino acids and carbohydrates (table 2.8). No amino acids were detected in the carbohydrate fraction and no carbohydrates were detected in the amino acid fraction eluted with $CaCl_2$, demonstrating quantitative separation of these analytes.

Table 3.6: Recoveries and correlation coefficients of amino acids

Amino acid	**% Recovery (peak areas)[a)]**	**Correlation coefficient [b)]**
Arginine	89.8	0.94
Ornithine	98.5	0.95
Lysine	96.0	0.96
Glutamine	93.2	0.84
Asparagine	93.7	0.94
Alanine	92.4	0.94
Threonine	96.5	0.96
Glycine	101.8	0.97
Valine	92.4	0.95
Serine	99.2	0.98
Proline	93.3	0.97
Isoleucine	105.7	0.97
Leucine	102.6	0.96
Methionine	109.6	0.98
Histidine	105.9	0.94
Phenylalanine	91.4	0.99
Glutamate	94.3	0.97
Aspartate	111.9	0.98
Cystine	114.8	0.95
Cysteine	125.8	0.95
Tyrosine	101.3	0.99

[a)] 42 nmol of each amino acid (0.21 nmol for cystine) was applied to the ion exchange resin, eluted with $CaCl_2$ / H_2O, and amino acids quantified by HPLC-IPAD. Results are means of 4 independent experiments.

[b)] 4.2 nmol, 21 nmol, 42 nmol, or 84 nmol of each amino acid (2.1 nmol, 10.5 nmol, 21 nmol, and 42 nMol for cystine) were subjected to the sample preparation protocol as above, amino acids quantified by HPLC-IPAD, and the amount of amino acids was plotted against the peak area. 2 to 4 independent experiments were carried out for each concentration and the experimental error was generally less than 10 % for all amino acids.

3.6.3 Detection of sugars and amino acids by IPAD

The resolution of the anion exchange separation is influenced by the sample matrix, column conditioning, and elution gradient. The separation by anionic exchange is highly sensitive to low pH values and high salt concentrations in the sample. Both factors result in poor resolution of many amino acids and in broadened peaks. The sample preparation described here results in two fractions containing carbohydrates and amino acids, respectively. The first fraction contains only low amounts of salts and methanol, and the analytes, namely sugars and sugar alcohols, are available in high concentrations. Samples typically may be diluted 10 to 50-fold prior to quantification by HPLC. In addition to carbohydrates, the fraction contains unknown matrix components; analytes which are eluted in the void volume, e.g. erythritol, can not be quantified. The second fraction with amino acids contains 150 mM $CaCl_2$, which may interfere with the subsequent quantification by HPLC. The complete elution of arginine and histidine requires high concentrations (200 mM) of $CaCl_2$. At the low concentration of amino acids typical for food samples, an exhaustive dilution is impossible. The effect of $CaCl_2$ concentrations in the sample on the separation of amino acids by anion exchange chromatography was evaluated using the amino acid stock solution adjusted to $CaCl_2$ concentrations from 0 to 200 mM. By injecting a series of gradually increasing concentrations of calcium chloride and applying an optimized gradient, we were able to determine that the maximal possible concentration of calcium chloride matrix is ca. 40 mM. As shown below (in the chromatograms of Figure 3.27, for example), concentrations equal or lower than 40 mM calcium chloride do not cause noticeable interference or peak distortions. Thus, food samples prepared according to the procedure proposed here (elution from ion exchange resin with 2 ml 200 mM $CaCl_2$ and 1 ml H_2O) must be diluted 5fold or higher prior to HPLC analysis, and appropriate amounts of food sample must be applied to the resin.

3.6.4 Validation of the sample preparation with food samples

In order to determine the suitability of the sample preparation procedure for food samples, four different food samples were analyzed with respect to carbohydrate and amino acid concentrations. Food were selected to analyze two carbohydrate rich samples (potato and sour dough), a fruit juice,

and a protein rich sample (milk). Two sample extraction methods were evaluated, sour dough, milk, lemon juice and potato were extracted with water, or with 7% perchloric acid to achieve de-proteinization of the samples. 100 µl supernatant were diluted with 100 µl methanol, 50 µl norleucine as internal standard, and 750 µl water, and were applied to extraction columns. Amino acids were eluted with 2 ml of a 0.2 M $CaCl_2$, 1 ml H_2O as described. It was verified by exhaustive washes of resins with 0.2 M $CaCl_2$ after the first elution, followed by HPLC analysis of the solvents, that amino acids were quantitatively recovered by the first elution step (data not shown).

The carbohydrate and amino acid fractions were collected, diluted 5fold, and the analytes were determined by HPLC. Furthermore, the unprocessed samples were diluted 5 fold and injected on the column. The chromatograms for carbohydrate and amino acid fraction as well as the unprocessed sample of sour dough extracted with water are shown in figure 3.27.

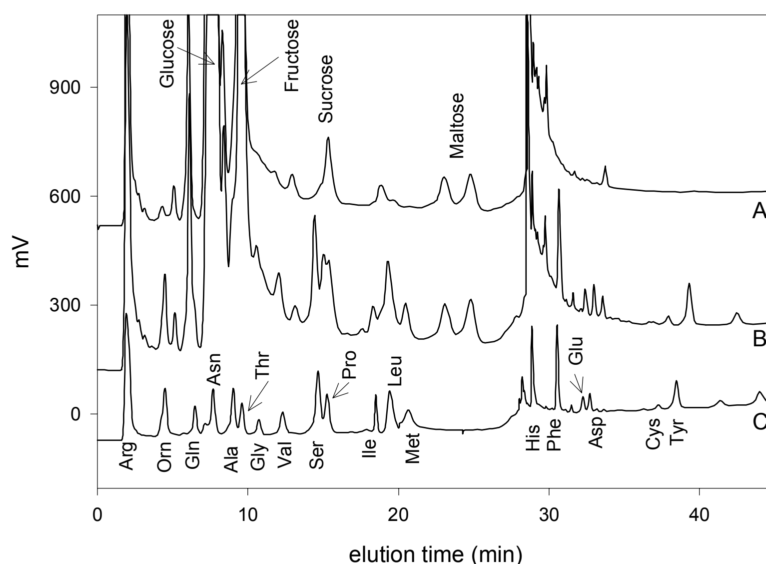

Figure 3.27: HPLC-IPAD separation of amino acids and carbohydrates in sour dough: A: Carbohydrate fraction of solid phase extraction containing sugars and sugar alcohols. B: Unprocessed sourdough sample after centrifugation and dilution. C: Amino acid fraction of solid phase extraction

In the unprocessed samples, most amino acids eluting during the first 16 minutes were obscured by sugars and sugar alcohols (figure 3.27 trace B, data not shown). Identification and quantification of those sugars employed in the carbohydrate standard was possible by analysis of the carbohydrate fraction of the food samples, erythritol eluted in the void volume. The carbohydrate fraction of the

aqueous extracts did not contain any amino acids, these were completely retained on the column. Upon de-proteinization of samples with perchloric acid, glutamate and aspartate could be detected in small but not negligible amounts in the carbohydrate fraction (data not shown). Additionally the perchloric acid does not allow the quantification of sucrose and other oligofructans because these are hydrolyzed to glucose and fructose in the presence of 3.5% perchloric acid. Thus, aqueous extraction was preferred.

The chromatograms of amino acid fractions obtained from aqueous extracts of sour dough, skim milk, lemon juice, and potato are shown in Figure 3.27 and Figure 3.28.

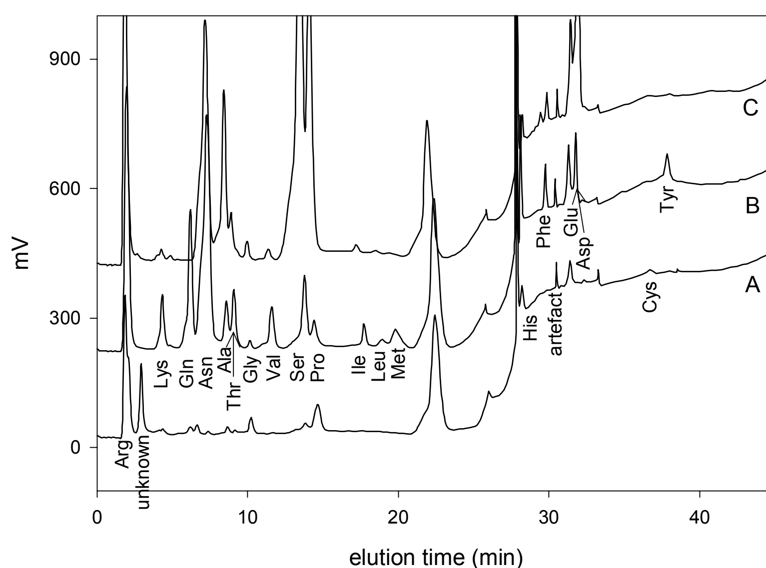

Figure 3.28: HPLC-IPAD separation of amino acid from food samples. Food samples were extracted and the amino acid fraction prepared as described (method 3). Norleucine was added as a internal standard to a final concentration of 0.5 mM. A: Skim milk containing 1.5 % fat. B: lemon juice. C: mashed potatoes.

After sample preparation, all amino acids can be identified. In the amino acid fraction from skim milk was detected one peak that could not be assigned to any of the amino acids for which the assay was calibrated. The recovery of norleucine used as internal standard was 98% (mean of 11 determinations, error of 10% or less), indicating quantitative recovery. To exclude losses of individual amino acids other than norleucine during sample preparation, the recovery of proteinogenic amino acids from food samples was determined for sour dough and lemon juice. 25 µM of each amino acid was added to the

samples prior to SPE, and the recovery was calculated based on the difference in amino acid concentration in the absence and presence of amino acids internal standard (table 3.7).

Table 3.7: Recoveries of amino acids from sour dough and lemon juice. Added were 25µM of amino acids to food samples, extraction was performed according to method 3.

	sourdough		**lemon juice**	
Amino acid	Δ Concentration (µM)[a)]	Recovery	Δ Concentration (µM)	Recovery
Arginine	24.5 ± 0.8	0.98	-	-
Alanine	25.1 ± 2.1	1.00	-	-
Threonine	24.6 ± 0.8	0.98	-	-
Serine	25.1 ± 1.3	1.00	-	-
Proline	24.6 ± 0.8	0.98	23.5 ± 2.6	0.94
Isoleucine	25.3 ± 0.7	1.01	27.0 ± 2.0	1.08
Leucine	25.4 ± 0.5	1.02	25.1 ± 2.1	1.00
Methionine	25.7 ± 0.7	1.03	24.7 ± 1.8	0.99
Histidine	25.5 ± 0.4	1.02	27.2 ± 0.4	1.09
Phenylalanine	23.0 ± 0.8	0.92	21.5 ± 2.8	0.86
Glutamate	25.5 ± 0.8	1.02	-	-
Aspartate	25.7 ± 0.7	1.03	-	-
Cysteine	- [b)]	[b)] -	21.9 ± 2.9	0.88
Tyrosine	23.4 ± 1.8	0.94	22.7 ± 2.3	0.91

[a)] Δ Concentration indicates the differences between amino acid concentrations in the absence and presence of amino acids internal standard. Values are means ± standard deviation of three independent experiments.

[b)] Amino acid recoveries were not calculated if the amino acid concentration in the food sample exceeded the amino acid levels originating from the internal standard more than 10fold.

Because the experimental error of extraction, sample preparation, and amino acid quantification by HPLC was in the range of 5 to 10% (table 3.7, data not shown), recoveries were not calculated when the amino acid content of the food sample exceeded the amino acid contents of the internal standard more than 10fold. Generally, the recoveries for amino acids were in the range of 90 to 108 %. Thus, recoveries of amino acids from foods are as quantitative as they were with standards solutions, and the

sample preparation described here is useful for quantitative analysis of amino acid and carbohydrate contents of food samples.

4. Discussion

4.1 Overview

Wheat sourdough is commercially used to obtain bread improved in flavor, texture and shelf live compared to “normal” bread. These changes are partly influenced by the wheat proteins and by their derivatives. These influences are shown in figure 4.1.

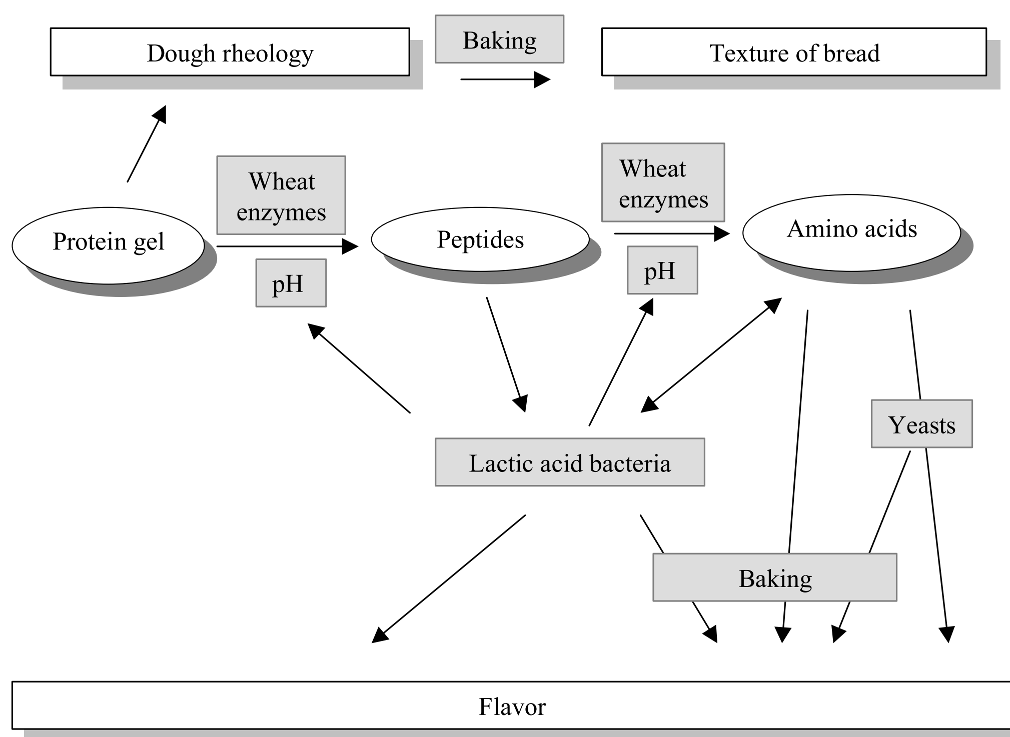

Figure 4.1: Influences of microorganisms, wheat enzymes and fermentation conditions on dough rheology and wheat bread texture and on the content of protein gel, peptides, amino acids and flavor compounds

During fermentation yeasts or lactic acid bacteria as well as endogenous wheat enzymes have a great influence on the composition and hydrolysis of gluten, peptides and amino acids. Fermentations with LABs, yeasts and under sterile conditions showed different amino acid contents which influenced the crust aroma. Doughs fermented with LABs or under sterile acidic conditions showed higher proteolytic activity increasing the flavor of wheat bread crust. For an additional improvement of flavor a screening on proteolytically active lactic acid bacteria was performed. No proteolytic strain was

found among 82 different strains. Yeasts were not included into this screening since they deplete the pool of free amino acids during exponential growth. In contrast to yeasts *L. sanfranciscensis* prefers the metabolism of peptides compared to amino acids and proteins. This was shown with nitrogen fractions isolated from fermented wheat dough. Degradation of protein gel to monomeric protein and peptides is mainly catalyzed by endogenous wheat enzymes. Microorganisms do not show any significant influence on the gluten macropolymer despite that they lower the pH during fermentation. This activates the wheat enzymes and leads to depolymerization and proteolytic breakdown of the protein gel. The development of a new method using FITC-labeled wheat proteins allows to distinguish between gliadin and glutenin which allows further insight into the gluten macropolymer during fermentations. Finally a new method that allows the determination of amino acid by IPAD from food samples was developed.

4.2 Generation of flavor precursor compounds during sourdough fermentation

We have determined the effect of acidification, addition of DTT, and sourdough fermentation on amino acid levels in dough. The results show that the amino acid levels in wheat doughs depend mainly on the pH of the dough, the fermentation time, and the consumption of amino acids by the fermentative microflora. It was demonstrated that increased levels of amino acids in doughs improved bread flavor. Furthermore, microbial formation of ornithine specifically enhanced the roasty note of bread crust odor.

The highest proteolytic activity was observed in acidified and reduced sterile doughs. Several mechanisms may account for the effect of salt, pH and reducing agents on the proteolytic system of wheat doughs. (i) The additives may effect the activity of proteolytic enzymes. Endogenous wheat proteinases have their optimum at a pH of 3.0 - 4.0 and the activity decreases towards higher pH (Kawamura, 1982, Wu and Hoseney 1989, Bleukx and Delcour, 2000). Reducing agents such as DTT may further activate sulfhydryl proteases which are present in wheat flour (Kruger et al., 1991). (ii) The release of amino acids is also influenced by the level of accessible substrate. The insolubility of gluten in water may limit the substrate concentration for proteolytic enzymes. The reduction of interprotein disulfide bonds during dough mixing enhances the solubility of gliadins and glutenins.

Lactic and acetic acids further swell gluten proteins. In our experiments, a reduced viscosity of reduced doughs was apparent immediately upon mixing whereas weakening of acidified doughs was observed only after 24 h of incubation. The addition of salt has an opposite effect on the solubility of gluten, because of the high sensitivity of gluten to salts (Preston and Kruger, 1976).

Dough fermentation with yeasts resulted in a decrease of free amino acids in the first fermentation phase due to microbial metabolism (Collar et al. 1991). *S. cerevisiae* and sourdough yeasts convert leucine and phenylalanine to the flavor volatiles 3-methylbutanol and 2-phenylethanol during dough fermentation (Hansen and Hansen, 1989; Damiani et al., 1996; Schieberle, 1996). Because of the high pH in the dough, the activity of flour proteinases is to low to replenish the amino acid pool and amino acid levels increase only after yeasts reach the stationary growth phase.

The enhanced proteolysis during sourdough fermentation is well in agreement with previous reports on the evolution of amino acid levels during sourdough fermentation (Collar et al., 1992; Gobbetti et al., 1994; Collar et al., 1991). Proteolysis in sourdough was mainly attributed to the proteolytic activity of *L. sanfranciscensis* and other sourdough lactobacilli (Gobbetti et al., 1996, Gobbetti et al., 1996b). In contrast, the comparison of amino acid levels in fermented and sterile acidified doughs presented in this work strongly indicates that the proteolytic activity of sourdough lactic acid bacteria does not exceed the demand of these organisms for amino acids during growth. Lactic acid bacteria require amino acids or peptides for growth (Juillard et al., 1998) and several amino acids are metabolized to flavor volatiles (Juillard et al., 1999, Smit et al., 2000, Tammam et al., 2000; Gao et al., 1998; Smacchi and Gobbetti, 1998). The observation that the uptake of nitrogen by lactobacilli did not result in reduced amino acid levels in doughs is explained by the preferential uptake of peptides and intracellular hydrolysis to amino acids (Aasen et al., 2000, Berg et al., 1981).

The levels of individual amino acids was influenced by pH and starter culture. Ornithine formation was observed only in doughs fermented with *L. pontis* because of the ability of this organism to convert arginine to ornithine (Vogel et al., 1994). Ograbek et al. (1999) demonstrated that the arginine metabolism of *L. pontis* was responsible for ornithine formation in wheat doughs and contributed to a more roasty flavor of the bread crust. Our results concur with these observations that ornithine formation in doughs is greatly enhanced by arginine metabolism of lactobacilli. Schieberle (1990)

reported that ornithine levels in wheat doughs and corresponding levels of 2-acetyl-1-pyrroline in wheat bread crust were enhanced by addition of bakers yeast. Ornithine levels in fresh bakers yeast range from 21 to 300 mg ornithine / 100 g yeast dry weight (Schieberle, 1990; Münch et al. 1997; Münch and Schieberle, 1998). We found virtually no ornithine in doughs fermented with yeasts only. These large differences are explained by the fact that bakers yeast usually is contaminated by lactobacilli that may or may not be able to convert arginine to ornithine. Based on a 5% addition of bakers yeast to wheat doughs it can be estimated that ornithine from bakers yeast accounts for less than 2 to 20 mg ornithine / kg dough. Addition at a level of 20% of a preferment prepared with arginine-degrading *L. pontis* provides greater 25 mg ornithine / kg dough (this study and Ograbek et al., 1999).

Accumulation of proline was favored by fermentation at near neutral pH, whereas release of hydrophobic and sulfur containing amino acids was greatly enhanced by chemical or microbial acidification. The pH for optimum activity of proline iminopeptidase apparently differs from that of other wheat proteolytic enzymes, which are mainly active at low pH (Bleukx et al., 1997).

The effect of proteolysis during dough fermentation of bread aroma was evaluated by baking experiments. Any preferment improved bread flavor. Preferments that were chemically acidified or fermented with *L. sanfranciscensis* enhanced the roasty odor of the bread crust, however, overall bread taste and flavor of the latter breads was superior to sterile preferments. The roasty note of bread crust odor was most pronounced in breads fermented with *L. pontis*. Because dough fermentation with *L. pontis* and *L. sanfranciscensis* resulted in comparable amino acid levels of the preferments with exception of arginine and ornithine levels, this roasty note may be attributed to the presence of ornithine in doughs fermented with *L. pontis*. It must be emphasized that the preferment application chosen in our study – addition of 5 – 20 % preferment to a yeasted bread dough – allows for two mechanisms by which amino acids are converted to flavor volatiles. (i) Conversion of amino acids to volatiles by baker's yeast during proofing of the bread dough and (ii) thermal degradation during baking (Schieberle, 1996). A notable exception is ornithine, which is not metabolized by *S. cerevisiae* but is converted to 2-acetyl-1-pyrroline ("roasty, cracker-like") during baking. We anticipate that the

differences between various preferments will be more pronounced in applications where the bread dough is leavened without addition of bakers yeast such as in traditional sourdough fermentations.

4.3 Screening of proteolytic lactic acid bacteria

For a further improvement of wheat sourdoughs a screening for lactobacilli with an increased proteolytic activity was done. A method based on the preculture of microorganisms in microtiter plates was developed. 82 different lactobacilli were screened on ca-caseinate agar and gluten agar. Preliminary experiments with unmodified recipes did not enable growth of tested lactic acid bacteria. After addition of some nutritients e.g. sugar and small amounts of yeast extract growth on both solid media was possible for 64 LAB. No strain showed any significant clearance on the used media and therefore no proteolytic activity was detectable. *Lactococcus lactis*, a strain with characterized proteolytic activity did not even show growth on these screening plates. The lack of proteolytic activity of LAB on solid media is in good accordance with observations of other work groups. Wiese (1995) tested the proteolytic activity of LAB on gluten plates, Wehrle et al. (1999) on gluten and casein plates with a subsequent Coomassie dye step and Mitra (1999) on agar containing either wheat albumin, wheat globulin or casein and on film strips containing gelatin. All experiments did not show any positive results when performed with LAB derived from sour dough. These tests on solid media are not suitable when the enzymes are either intracellular or connected to the cell wall so that they are not able to diffuse into the media to get contact to the proteins. In addition the test may be too insensitive for the low activity of cellwall bound proteinase.

A recent publication on the regulation of genes encoding proteolytic enzymes in *Lc. lactis* showed, that the addition of small amounts of peptides could repress the transcription of cellwall bound proteinase by 12 fold (Guedon et al., 2001). Due to the addition of small amounts of yeast extract to the modified media a repression of the proteolytic system is conceivable and could explain the negative results.

Proteolysis of LABs were tested based on the hydrolysis of FITC labeled proteins to obtain a more sensitive assay for proteolysis. Two *L. sanfranciscensis* strains were further investigated in the new

assay. Both microorganisms were isolated from industrial processes and one of them, ATTC 2765, is known as proteolytically active sourdough strain (Brandt, personal communication). The assay showed a high sensitivity for commercially available papain and fungal protease as well as for *B. subtilis*. For both lactobacilli strains no activity was detectable, neither with casein nor with cereal proteins, although both were metabolic active and metabolized sugar to lactic and acetic acid. These assays contained no known peptide and amino acid source except for the labeled proteins, a repression of proteinase can be ruled out. Wehrle et al. (1999), Di Cagno et al. (2002) and Mitra (1999) were able to show proteolytic degradation of cereal proteins by *L. sanfranciscensis* in liquid media. A proteolytic activity of the chosen strains should be detectable. It could be that the fluorescence prevents the access of the protein to the active site of the protease or that the cells are able to take up the amino acid FITC complex after the cleavage and compensate therefore the quenching effect.

L. sanfranciscensis grows in sour dough, which contains high quantities of free amino acids, peptides and proteins. A proteolytic system to additionally degrade wheat protein is not required and it can be suggested, that the expression of an extracellular protease would be repressed under these conditions of amino acid and peptide abundance. Nevertheless the principles of the uptake of amino nitrogen in *L. sanfranciscensis* is still unclear.

4.4 Nitrogen demand and peptide utilization by *L. sanfranciscensis*

From other lactic acid bacteria it is wellknown, that they require many nutrients in the growth media, including amino acids (Morishita et al., 1981). In a review prepared 1996 by Kunij et al. it was shown for *Lc. lactis* as well as for a variety of lactobacilli that they have transport enzymes for the uptake of peptides and the intracellular degradation of peptides to amino acids (see also Christensen et al., 1999). It seems, that the transporters and enzymes are not identical but similar within the different lactic acid bacteria. There are differences in the amino acid auxotrophies and in the size of the peptides which are assimilated, e. g. *Lc. lactis* can use peptides up to 13 amino acid residues and *Leuc. mesenteroides* only peptides up to 7 amino acids (Foucaud et al., 2001).

For *L. sanfranciscensis* a peptide isolated from yeast extract was found which enhances growth in a medium with low amino nitrogen content. It has a molecular weight of about 1065 Da and consists of

asp, cys, glu, gly and lys (Berg et al., 1981). In this study it should be determined whether this is an exception or if *L. sanfranciscensis* prefers uptake of peptides in general.

Wheat dough fermented with *L. pontis*, *S. cerevisiae* and an aseptical acidified dough were extracted and fractionated by size exclusion chromatography and cation exchange chromatography. These fractions were added to an amino nitrogen limited growth media and always the same amount of amino nitrogen (determined as UV adsorbance) was added. Growth of *L. sanfranciscensis* was enhanced by several fractions and all of these fractions had in common, that they contained peptides. It was a preference in amino nitrogen uptake detectable. Growth was not significantly improved by fractions containing low molecular weight molecules like amino acids or by fractions containing only proteins.

In sourdough fermentations with lactic acid bacteria it was detected, that these doughs showed the same overall content in amino acids compared to aseptically acidified control doughs. There was no depletion of amino acids detectable although the micro-organisms needed amino nitrogen for cell growth. This is now explainable because the need for amino acids was met by the uptake and the intracellular hydrolysis of peptides.

The mechanism of peptide uptake was elucidated in the last few years. Strobel et al. (1989) and Nakajima et al. (1998) reported a proton motive force dependence of amino acid transport in *L. casei* and *L. helveticus* , respectively. In *L. helveticus* NCDO2712 the essential amino acids proline, phenylalanine and tryptophan could not be internalized when they were provided only as amino acids (Nakajima et al., 1998). Mutations of the oligopeptide transporter system of *Lc. lactis* , which disabled a translocation of the peptides, resulted in a very low concentration of some amino acid in the cytosol (Kunij et al., 1995).

The uptake of peptides instead of amino acids in several food ecosystems is of great advantage for micro organisms. In milk almost no amino acids are available, the amino nitrogen demand is met to 90 % by the proteolytic breakdown and the uptake of the resulting peptides (Flambard, 1998). In meat the role of proteolytic breakdown is different. The proteolytic activity is mainly based on endogenous muscle enzymes like cathepsins and the microorganisms are mainly confined to the secondary hydrolysis of oligopeptides and small peptides (Fadda et al., 1999). The role of lactic acid bacteria in

sourdough is still not absolutely clear, it was shown in this work that *L. sanfranciscensis* does not show any proteolytic activity under the circumstances that were used and that this microorganism meets its amino nitrogen demand by the uptake of peptides. On the other hand it was shown that a wheat dough fermentation with yeast leads to a compete depletion of amino acids during the exponential growth phase.

4.5 Depolymerization and proteolytic breakdown during sourdough fermentation

In this study was shown that fermentation of wheat doughs in the presence of organic acids results in virtually quantitative depolymerization of the glutenin macropolymer (GMP) and partial hydrolysis of glutenin subunits. Differences observed between neutral and acid control doughs with respect to glutenin depolymerization and proteolytic degradation of individual HMW glutenins were more pronounced than those differences between doughs fermented with *Lactobacillus sanfranciscensis* or *Lactobacillus pontis*. Major effects are therefore attributable to the acidity and proteolytic enzymes of wheat flour rather than proteolytic activities of sourdough lactobacilli.

The fermentation conditions had no major effect on proteolytic events as determined by the release of amino nitrogen. Using the same strains as studied in this work, but a different flour, Thiele et al. (2002) reported that comparable levels of amino nitrogen and total amino acids were found after 24h of fermentation at acid or neutral conditions, or in sourdoughs started with lactobacilli. Sourdoughs and acid control doughs showed increased amino nitrogen levels after 48 h of fermentation compared to neutral doughs and the concentration of individual amino acids indicated that different substrates are hydrolyzed under neutral and acidic conditions (Thiele et al., 2002). Lactobacilli have multiple amino acid auxotrophies and oligopeptide transport is considered the main route for nitrogen entry into the bacterial cells (Kunji et al., 1996). Berg et al. (1981) have isolated a peptide with a MW of 1065 containing aspartate, cysteine, glutamate, glycine and lysine that stimulates growth of *L. sanfranciscensis*. In our study it was shown *in situ* during sourdough fermentation that *L. sanfranciscensis* and *L. pontis* utilized peptides to meet their nutritional requirements with respect to amino acids.

Based on the levels of amino nitrogen generated by proteolytic events during fermentation, it can be estimated that less than 5% of wheat proteins were hydrolyzed during fermentation. Di Cagno et al. (2002) reported that proteolytic degradation of wheat proteins during sourdough fermentation results mainly in the degradation of albumins, globulins, and gliadins. Because proteinases of lactobacilli are bound to the cell wall (Siezen, 1999), they must be assumed to have only limited access to the insoluble GMP. In our study was not detected a decrease of gluten proteins (sum of gliadins and glutenins) during fermentation. However, individual glutenin subunits were hydrolyzed during sourdough fermentation to proteins with lower molecular weight that remained associated with the gluten macropolymer. Bleukx et al. (1997) observed that autodigestion of Osborne fractionated gluten at acidic conditions resulted in glutenin hydrolysis and the generation of degradation products. Similar protein patterns as those reported by Bleukx et al. (1997) were observed by electrophoretic analysis of Osborne fractions obtained from fermented doughs. The comparison of acid control doughs with sourdoughs fermented with or without addition of phosphate buffer indicated that hydrolysis of glutenins mainly depended on the pH and was not related to specific proteinases of lactic acid bacteria, in accordance with previous observations that wheat proteinases degrading gluten proteins have their optimum activity at pH values of 4.0 or below (Wu and Hoseney 1989, Bleukx and Delcour, 2000).

Sequential extraction of doughs with SDS / SDS-DTT or propanol / propanol-DTT has shown that glutenin polymers were depolymerized during fermentation of acid control doughs and doughs fermented with *L. sanfranciscensis* or *L. pontis*. Increased amounts of protein were extracted with SDS or propanol; correspondingly, decreased amounts of proteins were recovered in the second extraction using SDS-DTT or propanol-DTT. Gluten depolymerization was furthermore apparent upon SEC analysis of SDS-soluble proteins as the increased amounts of protein extracted with SDS were attributable mainly to monomeric protein. After 24 h of fermentation, the distribution of proteins in propanol and propanol-DTT sequential extracts qualitatively corresponded to their distribution in SDS / SDS-DTT extracts. Several factors may account for the gluten depolymerization, the enhanced solubility of glutenins at low pH in the presence of organic acids, proteolytic degradation of glutenin subunits, and inhibition of glutathione dehydrogenase by low pH.

The concentrations of lactic and acetic acids of wheat sourdoughs after 24h of fermentation typically range between 150 to 250 and 20 to 50 mM per kg dough, respectively, well in the range of acid levels known to enhance solubility of glutenins. The pH affects non covalent interactions between glutenin subunits and thus may increase their SDS solubility. SDS-soluble and insoluble glutenin polymers are thought to differ in their molecular conformation in addition to their molecular weight (Carceller and Aussenac, 2001). However, the buffering capacity of extraction solvents used in this work compensated differences in pH of the various dough samples. Especially during sequential extraction of salt, propanol, and propanol DTT soluble proteins, care was taken to remove organic acids originating from the doughs prior to the propanol and propanol-DTT extraction steps. Proteolytic degradation of glutenins does not account for the glutenin depolymerization apparent already in unfermented acid control doughs, because degradation of glutenin subunits was observed only after 6 and, more pronounced, after 24h of fermentation.

Sulfhydril / disulfide (SH / SS) interchange reactions of low molecular weight sulfhydryls into flour proteins occur during dough mixing and weaken the gluten by SS-interchange of intermolecular SS bonds (Grosch and Wieser, 1999, Hahn and Grosch, 1998). The observation that the ratio of SDS / SDS-DTT as well as the ratio of propanol / propanol-DTT soluble proteins was strongly increased during sourdough fermentations, as well as acid control fermentations, indicate that reduced intermolecular disulfide bonding of glutenin proteins contributed to the GMP depolymerization. Upon inhibition of GSH dehydrogenase by low pH during dough mixing (Kaid et al., 1997), GSH may remain available for glutenin depolymerization through disulfide interchange. Lactic acid bacteria strongly reduce the redox potential of wheat doughs during fermentation (Hammes and Gänzle, unpublished), furthermore, the activity of glutathione dehydrogenase of lactic acid bacteria (Bolotin et al., 1999) may contribute to depolymerization of disulfide bonded glutenins.

In wheat sourdough processes typical for the bakery practice, 10 to 20% of the flour are fermented to a dough pH of 3.6, whereas the major part of the flour is fermented only for short times (< 3 h) and the pH of the bread dough is typically between 4.0 and 4.5 (traditional processes) or > 4.5 (preferment addition to straight dough processes). Thus, depending on the type of sourdough fermentation process

employed, 10 % or more of the glutenin macropolymer is hydrolyzed and / or depolymerized during fermentation, resulting in a weakened gluten network in bread doughs. The literature data available on loaf volumes of sourdough fermented wheat breads demonstrates that increased volumes are obtained through sourdough fermentation when compared to straight dough processes under otherwise identical conditions (Corsetti et al., 2000, Corsetti et al., 1998, Collar Esteve et al., 1994; Salovaara and Spicher, 1987). The conclusion that, of all flour components, protein or protein related parameters determine bread-making qualities to the greatest extent (Weegels et al., 1996a) may therefore not apply for sourdough fermented wheat breads. Our study indicates that gluten properties of wheat flours are of decreased importance in sourdough baking whereas other factors are expected to be more relevant. Wheat arabinoxylans are solubilized during sourdough fermentation, and sourdough lactobacilli produce substantial amounts of exopolysaccharides during fermentation (Martinez Anaya and Devesa, 2000, Korakli et al., 2001). The increased contents of water soluble polysaccharides in sourdoughs fermented bread doughs compared to straight doughs contributes to the water absorption and gas retention capacities of the doughs as observed in rye baking. Changes in the wheat polysaccharides rather than changes in wheat proteins are likely to explain the beneficial effect of sourdough fermentation on loaf volume of wheat breads.

4.6 Depolymerization and proteolytic breakdown of FITC-Proteins

Sourdough fermentations are known to affect wheat proteins which influence dough rheology, flavor and bread texture. In previous work the release was investigated of amino acids (Collar et al., 1991, Gobbetti et al., 1996, Thiele et al., 2002) and the hydrolysis of proteins during sourdough fermentation (di Cagno et al., 2002). So far it was not possible to observe in situ distinct protein fractions during fermentation in food matrices. In this study a new method was described providing information on the hydrolysis and functional properties of proteins during fermentations in wheat dough.

Fractions of albumin/globulin, gliadin, and glutenin were labeled with FITC and after purification these fractions were inoculated into different wheat doughs. The labeling and purification of fractionated wheat proteins with FITC was performed essentially according to Twining (1984). Modifications of this method were required to prevent precipitation of proteins with low solubility in

aqueous solutions, and to completely remove unbound FITC. The latter is of prime importance when labeled proteins are incorporated in a complex matrix, because unbound FITC reacts with all free amine groups. The labeled wheat proteins fractions (albumin/globulin, gliadin and glutenin) that contained enough fluorophore to be detected by SEC coupled to a fluorescence detector. No differences in molecular size was detectable when native proteins were compared with FITC-labeled proteins by SDS-PAGE.

Wheat proteases degraded all FITC labeled proteins partially to peptides and amino acids. In aseptic, neutral doughs the release of peptides and amino acids was very low. In doughs acidified either from beginning or during microbial fermentation, an increased release of low molecular weight fluorescence was observed. Several proteases from wheat endo- and exosperm have been characterized (Bleukx et al, 1997) and several of them have their maximum activity at low pH values (Bleukx and Delcour, 2000). Degradation was observed not only of the water soluble substrates albumins and globulins, but also the water insoluble gliadins and glutenins were hydrolyzed. There is no evidence for an increase in free FITC due to enzyme activity because the HPLC retention time of fluorescence labeled low molecular weight amino acids and peptides differs from that of unbound FITC.

Compared to the proteolysis in aseptic acidified doughs, the additional proteolytic activities of microbial enzymes was small. It was previously observed that lactobacilli have little effect on total amino acid concentrations in wheat sourdoughs when compared to acid aseptic doughs, whereas fermentations with yeasts significantly decrease amino acid concentrations (Thiele et al., 2002). However, whereas microbial enzymes do not influence to a great extend the overall degradation of proteins, and the accumulation of amino acids, the hydrolysis of individual wheat proteins was affected by fermentation with strains of lactobacilli selected for their high proteolytic activity (di Cagno et al., 2002). In this study was found that microbial fermentation affected the size distribution of peptides resulting from proteolytic degradation of wheat proteins. When fermented without microorganisms, the majority of low molecular weight fluorescence is found as peptides. In the presence of lactobacilli, the concentration of larger peptides decreased and that of smaller molecules

such as dipeptides and amino acids, increased, whereas the overall amount of low molecular weight fluorescence remained unaltered.

The proteolytic system of lactic acid bacteria consists of cell-wall associated proteinases which convert proteins to oligopeptides (Siezen, 1999). Di-, tri- and oligopeptide transport is the main route for nitrogen entry into the bacterial cells and virtually all peptidases are located intracellularly (Gobbetti et al., 1996, Kunij et al., 1996) and uptake of peptides is preferred over amino acid uptake (Foucaud et al., 2001). Studies on the regulation of proteolytic activity of lactic acid bacteria indicate that proteinase activity is essentially absent when the organisms are growing on substrates rich in peptides (Guédon et al., 2001; Hebert et al., 2000). The proteolytic system of *L.* sanfranciscensis compares well to that of other lactic acid bacteria (Gobbetti et al., 1996) and Berg et al. (1981) isolated a peptide from yeast extract which enhances growth of *L. sanfranciscensis*. Our data suggest that lactobacilli in sourdough fermentations are not only capable to growing on peptides as the only source for essential amino acids but prefer the uptake of peptides over amino acid transport.

The addition of a fungal protease to a aseptic acidified dough increased the overall amount of peptides and amino acids but failed to fully hydrolyze monomeric as well as polymeric proteins. When protease was added to *L. pontis* sourdoughs, a strong degradation of almost 90% of proteins was detectable. This synergistic effect of protease and lactic fermentation is not explained by acidification. The concentration of peptides is higher in co fermented doughs than in doughs containing only the protease, excluding shifts in the reaction equilibrium through removal of products by lactobacilli.

The labeled proteins exhibited functional properties corresponding to those properties of native proteins. Labeled glutenin and gliadin became partially incorporated into the protein gel during fermentation. Incorporation of FITC labeled model prolamin and glutelin into the protein gel was successfully performed by Lindsay et al. (2000). Hordeins containing zero, one or two additional cysteine residues were designed through genetic engineering and it was shown, that polymerization of these hordeins is strongly dependent on the amount and the position of cysteine residues in each protein molecule. In this study was observed the incorporation of FITC labeled gluten proteins and major effects on the incorporation of FITC-proteins were attributable to the pH. Glutenins were only incorporated into SDS-soluble polymers and the SDS-insoluble GMP at neutral pH and the formation

of the GMP was inhibited at low pH. The solubility of gluten polymers is not only influenced by the molecular size but also by the density of the molecules (Carceller and Aussenac, 2001). FITC-gliadin was almost exclusively found in the SDS soluble protein fraction. Two hypotheses may explain the integration of FITC-gliadin into the polymeric protein fraction: (i) the gliadin fraction contained not only gliadins but also small amounts of LMW-glutenins (ii) gliadin is incorporated into the protein polymer either by a covalent linkage due to an intermolecular mismatch of cysteine-residues or by noncovalent interactions between polymeric proteins and gliadin. Proteins containing only one free cysteine, e.g. some classes of LMW-glutenin residues, act as chain terminator (Lindsay and Skerritt 1999, Lindsay et al., 2000). Proteins in the FITC labeled gliadin fraction with one sulfhydryl moiety available for intermolecular bonding would lead to fluorescence mainly in smaller and therefore soluble protein polymers as observed in this study.

In acid control doughs, immediately after dough mixing less polymeric proteins were observed compared to neutral doughs. During resting time, no integration of FITC-glutenins or FITC-gliadins into the GMP was detectable in acid control doughs. Doughs fermented with lactobacilli showed two different phases with respect to gluten polymerization. During the first 7 hours, corresponding to a dough pH of 4.5 or higher, FITC- gliadins as well as FITC-glutenins were incorporated into the polymeric protein fraction and / or the SDS-insoluble GMP. After a decrease of the pH to < 4.0, polymerization stopped and fluorescent proteins which were already incorporated into the GMP were released during fermentation. After 24 hours the overall amount of monomeric proteins in sourdoughs were comparable to the content in acid control dough. Differences between doughs fermented with *L. pontis* and *L. sanfranciscensis* are attributable to different lag times and acidification capacities of the two organisms and major effects concerning gluten (de-)polymerization are attributable to the pH.

The labeling of proteins with FITC and the subsequent addition to complex matrices is not only practicable in dough but may furthermore be useful for other fermented foods such as fermented meats or cheese where proteins are modified or degraded during fermentations. It can provide *in situ* information on the fate of single protein types during fermentation and on protein fractions which originate from degradation. Additionally, the detection limit of FITC is very low, only very small amounts of labeled proteins must be incorporated, which prevents artifacts due to protein addition.

4.7 Amino acid determination by IPAD

A method for the determination of single amino acids was needed to elucidate the metabolism in wheat sour dough. A solid phase extraction protocol was established for the quantification of amino acids and carbohydrates from food samples by HPLC-IPAD. This method is fast and flexible and does not require dedicated equipment for sample preparation. Diluted samples are added to strong cation exchange columns, and carbohydrates are recovered in a first elution step. Amino acids are eluted with 0.2 M $CaCl_2$ in a second step. Recovery values for all amino acids, including arginine, aspartate, and glutamate, were in the range of 90% and 126%. Application to the sample preparation of the 21 amino acids in various concentrations resulted in calibration curves with correlation coefficients 0.84 and 0.99. The method was validated for food samples.

5. Conclusions

The aim of this work was to determine the effects of proteolytic events and bacterial amino acid metabolism on bread quality. The influences of microorganisms, wheat enzymes and environmental conditions on the gluten macropolymer, the hydrolysis of wheat proteins and peptides and the release of amino acids should were elucidated.

Fermentation of wheat doughs either with lactic acid bacteria or with yeast showed clear differences in amino nitrogen levels of doughs. During exponential growth yeasts depleted the overall amount of amino acids remarkably and within stationary growth the amino acid pool was only partially refilled. This quality made yeasts uninteresting for further investigations on amino nitrogen demand because it was clearly shown, that they will reduce the amount of flavor precursor during fermentation time. In contrast to this observation *L. pontis* as well as *L. sanfranciscensis* did not show any significant amino acid uptake. Doughs fermented with LABs had about the same amino acid content as doughs fermented sterile (acid or DTT were additionally added). They improved the overall crust flavor and doughs fermented with *L. pontis* showed best results especially for the roasty note.

For a further improvement of bread flavor, a higher amount of amino acids is preferable and therefore a screening for lactic acid bacteria with a higher proteolytic activity was performed. 82 different strains were screened on solid media. No tested strain showed any activity under the used conditions. This result was explained either by a too low sensitivity of the tests or by regulatory repression of the cellwall bound protease due to small amounts of peptides available in the media.

To determine how LABs meet their amino nitrogen demand, fermented dough samples were extracted and separated by HPLC to gain samples containing proteins, peptides and amino acids. It was shown that peptides were able to improve growth of *L. sanfranciscensis* on medium limited in amino nitrogen. Based on additional informations it was concluded that lactobacilli prefer the uptake of peptides which are subsequently hydrolyzed in the cytosol.

Further investigations were performed on the influences of lactobacilli and endogenous wheat enzymes on wheat proteins. The results indicate that sourdough fermentation has a major impact on gluten quality. Depolymerization and hydrolyzation were observed and could be attributed mainly to

low pH cereal proteases. During fermentation the pH is lowered from about 6.2 to 3.6 by microbial metabolism and this leads to an improved proteolytic breakdown. Additionally we suppose that peptides generated by cereal enzymes are subsequently taken up by lactic acid bacteria to meet their amino nitrogen demand. And the amino acids remain in the dough and are still available for yeast or in our case as precursor for a more intense wheat crust flavor.

6. Zusammenfassung

Ziel dieser Arbeit war es, den Einfluss von proteolytischen Vorgängen und bakteriellem Aminosäurestoffwechsel auf die Qualität von Weizengebäcken zu bestimmen. Deshalb wurde der Einfluss von Mikroorganismen, Getreideenzymen und Teigführung auf den Kleber, den Abbau von Weizenproteinen und Peptiden sowie die Freisetzung von Aminosäuren untersucht.

Die Fermentation von Weizenteigen mit Milchsäurebakterien oder Hefen zeigte deutliche Unterschiede in der Aminosäureaufnahme. Hefen verbrauchten fast die gesamte Menge an freien Aminosäuren während des exponentiellen Wachstums, die Aminosäurekonzentration nahm auch während der stationären Wachstumsphase nur langsam zu. Dieser signifikante Verbrauch an Aminosäuren und damit an Aromastoffen führte dazu, dass alle weiteren Versuche mit Laktobazillen durchgeführt wurden. In Fermentationen mit *Lactobacillus pontis* und *L. sanfranciscensis* konnte kein signifikanter Aminosäureverbrauch festgestellt werden. Diese Teige zeigten in etwa die gleiche Aminosäurefreisetzung wie sterile Teige, die entweder angesäuert oder reduziert wurden. Sowohl die angesäuerten sterilen Teige als auch die mit Milchsäurebakterien fermentierten Teige konnten das Aroma von Weizenbrötchen verbessern.

Zur weiteren Verbesserung des Brotaromas sollte die Ausbeute an freien Aminosäuren erhöht werden. Dazu wurden ein Screening von 82 verschiedenen Milchsäurebakterien durchgeführt, um Stämme mit erhöhter proteolytischer Aktivität zu finden. Auf den verwendeten Agarplatten konnte keinerlei Aktivität festgestellt werden. Entweder waren die verwendeten Testsysteme zu wenig sensitiv für die zellwandgebundene Protease, oder aber es lag eine Hemmung der Proteasesynthese vor. Dies kann auf die im Medium, zwar nur in geringen Mengen vorhandenen Peptide zurückgeführt werden.

Die Tatsache, dass *L. sanfranciscensis* seinen Stickstoffbedarf nicht über freie Aminosäuren deckt, wirft natürlich die Frage auf, was er statt dessen nutzt, um die vorhandenen Auxotrophien zu decken. Dazu wurden fermentierte Weizenteige extrahiert und mittels HPLC der Grösse nach aufgetrennt, um Fraktionen mit Proteinen, Peptiden oder Aminosäuren zu erhalten. Es wurde gezeigt, dass vor allem Peptide das Wachstum von L. sanfranciscensis auf einem stickstofflimitierten Medium verbessern konnten. Weitere Informationen aus der Literatur unterstützen die Vermutung, dass Laktobazillen die

Aufnahme von Peptiden sowie deren anschließender Hydrolyse im Cytosol der Adsorption von freien Aminosäuren vorziehen.

In den durchgeführten Teigversuchen konnten Veränderungen an der Teigstruktur und an der Teigzusammensetzung festgestellt werden. Die Einflüsse von Laktobazillen sowie der Weizenenzyme auf die im Mehl enthaltenen Proteine wurden daraufhin untersucht. Als Vergleichsteige dienten angesäuerte sterile Teige. Die Ergebnisse zeigten, dass der Abbau von Weizenkleber nicht direkt auf die Mikroorganismen zurückzuführen ist. Sowohl die Depolymerisierung als auch der proteolytische Abbau der Proteine ist vor allem auf die weizeneigenen Enzyme zurückzuführen. Diese Enzyme werden durch eine Ansäurung des Teiges aktiviert, und hier kommen auch die Laktobazillen ins Spiel. Während der Fermentation werden durch die Milchsäurebakterien organische Säuren gebildet und dadurch sinkt der pH Wert von ca. 6.2 auf 3.6, was die verstärkte Depolymerisierung im Vergleich zu neutralen Teigen ermöglicht. Die gebildeten Peptide werden dann von den Laktobazillen zur Deckung des Stickstoffbedarfs aufgenommen. Die Aminosäuren verbleiben im Teig und dienen als Vorstufe für das Krustenaroma, oder als Nährstoffe für Hefen.

7. References

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8. Appendix

List of papers that resulted from this Dissertation:

Thiele, C., Gänzle, M.G., and Vogel, R.F. (2002) Contribution of sourdough lactobacilli, yeast, and cereal enzymes to the generation of amino acids in dough relevant for bread flavor. Cereal Chemistry 79(1): 45-51.

Thiele, C., Gänzle, M.G. and Vogel, R.F. (2002) Sample preparation for amino acid determination by integrated pulsed amperometric detection in foods. Analytical Biochemistry. 310(2): 171-178.

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Lebenslauf

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