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Fluorescence Labeling of Wheat Proteins for Determination of Gluten Hydrolysis and Depolymerization during Dough Processing and Sourdough Fermentation

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This study was undertaken to enable the determination of hydrolysis and functionality of proteins in situ during fermentation of wheat doughs. Wheat proteins were fractionated and labeled with fluorescein-isothiocyanate (FITC). Fluorescent proteins were incorporated into wheat sourdoughs inoculated with lactobacilli and into neutral and acid control doughs. Doughs containing fungal protease were furthermore evaluated. Doughs were analyzed by extraction and size exclusion chromatography analysis of sodium dodecyl sulfate soluble proteins. Labeled proteins exhibited characteristics comparable to native proteins, with respect to proteolytic degradation and polymerization. Proteolytic breakdown of proteins was enhanced at low pH. Glutenin subunits were incorporated into the gluten macropolymer at neutral pH. Polymerization of FITC proteins was not observed at low pH. Sourdoughs were comparable to acid control doughs, major effects were attributed to changes of pH, rather than microbial metabolism. A synergistic effect with respect to proteolytic activity was observed between fungal protease and *L. pontis*.

KEYWORDS: Gluten; FITC; polymerization; proteolysis; sourdough; *Lactobacillus sanfranciscensis*; *Lactbacillus pontis*

INTRODUCTION

The quality of fermented foods is influenced by functional properties of proteins as well as their degradation products (*1*−*4*). During fermentation, proteins are partially degraded to peptides and amino acids, and these proteolytic events contribute to the taste, flavor, and texture of foods, make them more appealing, and affect the shelf life. Amino acids and peptides themselves affect the taste of fermented foods; furthermore, they are converted to flavor volatiles by microbial metabolism or by thermal reactions, e.g., during baking. The texture of the products is determined by protein functionality, i.e., solubility or protein cross-linking by covalent and noncovalent interactions. Thus, the analysis of proteolytic events for optimization of food fermentation processes requires not only quantification of proteolysis products but also analysis of individual proteins which serve as substrate for degradation.

Sourdough fermentation retained its importance in bread production, because it improves the flavor, loaf volume, texture, and shelf life of bread (*5*−*7*). Flavor is influenced by microbial modification of amino acids during fermentation and thermal degradation during baking (*8, 9*). Ornithine, proline, isoleucine, phenylalanine, and methionine, in particular, are precursors for flavor active compounds. Accordingly, proteolytic events during sourdough fermentation were shown to improve bread flavor (*7*). The hydrolysis of proteins in sourdoughs is attributable to cereal proteases (*7, 10, 11*), and sourdough lactic acid bacteria selected for their high proteolytic activity also contributed to the hydrolysis of wheat proteins in a strain specific manner (*12*).

The rheology of wheat doughs in straight dough processes and the resulting loaf volume of bread are mainly determined by gluten proteins. The bread making quality of wheat flours is correlated to the overall protein content, but is also governed by the protein composition that influences the interactions between proteins and the structure of gluten polymer (*13*). Glutenin subunits form the glutenin macropolymer (GMP), large polymers with molecular masses up to several million. This protein aggregation is caused by intermolecular disulfide bonding and tyrosine cross-links between the glutenin subunits and by non covalent interactions (*14*−*16*). The amount of gel protein or GMP, which is insoluble in diluted acetic acid or sodium dodecyl sulfate (SDS), correlates well to the loaf volume (*17*, *18*). The supernatant contains all SDS-soluble proteins, including albumin, globulin, gliadin, and glutenin polymers not associated with the GMP. A widely used technique for the characterization of the size distribution of glutenin polymers is high-performance size exclusion chromatography (SEC) (*19*, *20*).

Although an increased understanding has been achieved in recent years concerning the influence of glutenin subunits and GMP on bread quality, only few data are available on the behavior of wheat proteins during sour dough fermentation. Investigations on the rheology of sourdough demonstrate a weakening of the gluten network during fermentation (*21, 22*).

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Table 1. 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	390	200	200	390	200
culture (μL)[a]			200	200		200
chloramphenicol 10% w/ vol (μg)	2	2			2	
FITC–protein or water (μL)[b]	100	100	100	100	100	100
protease (mg)[c]					1.7	1.7
acid (μL)[d]		8			8	

[a] Approximately 10^9 cells of *L. pontis* TMW 1.397 and *L. sanfranciscensis* LTH2581, respectively. [b] The following protein additions were used in all doughs: FITC–albumin/globulin, FITC–gliadin, FITC–glutenin, and addition of water served as control. [c] Fungal protease commercially available for use in baking improvers. The protease preparation contains a mix of proteinase and peptidase activities prepared from *Aspergillus oryzae* and has a specific activity of 100 haemoglobin units g^{-1} (acid protease on haemoglobin substrate). [d] Mixture of 4 volumes of lactic acid (90%) and 1 volume of acetic acid (98%) to obtain a dough pH of 3.5.

Proteolysis in wheat doughs was previously determined by quantification of amino acids (*7, 23*) but did not allow determination of whether those gluten proteins relevant for the rheological properties of wheat doughs were substrates for hydrolysis. To determine the substrates of proteolysis, dough extracts were fractionated and analyzed by 2-dimensional (2D) gel electrophoresis (*12*). However, on the basis of the amino acid levels of sourdough, it can be estimated that less than 5% of total wheat proteins are degraded. Thus, changes of the concentrations of individual proteins during fermentation are within experimental error of most analytical methods used for protein quantification. Furthermore, analysis of the amount of a given protein does not provide information on size and functionality of the degradation products. It was, therefore, the aim of this work to provide a method for in situ analysis of qualitative and quantitative changes of the wheat protein fractions albumin and globulin, gliadin and glutenin during wheat dough fermentations. Experiments were carried out to label extracted wheat protein fractions with the fluorescent dye fluorescein-isothiocyanate, (FITC)(*24*), and to follow the degradation and funcitonality of FITC-labeled proteins in reconstituted wheat doughs and wheat sourdoughs.

MATERIALS AND METHODS

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 essentially performed according to Kruger et al. (*25*) with the following solvents: (**1**) 0.5 M NaCl, 150 mM sodium phosphate, pH 6.8, to obtain the albumin and globulin fraction; (**2**) 50% *n*-propanol in H_2O_{bidest}, to obtain the gliadin fraction, (**3A**) 50% *n*-propanol in H_2O_{bidest}, 1% acetic acid and 4% DTT, to obtain a monomeric, reduced glutenin fraction. As an alternative to solvent **3A** containing dithiothreitol (DTT), glutenins were extracted in the presence of SDS without DTT to obtain a polymeric, unreduced glutenin fraction: (**3B**) 1.5% SDS in 50 mM sodium phosphate buffer, pH 6.9. To 10 g of wheat flour was added 40 mL of solvent, and 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 with 20 mL of either solvent **3A** or solvent **3B**. All extracts were stored at −20 °C.

Labeling with FITC and Purification of Labeled Proteins. Labeling with FITC was performed essentially according to Lindsay et al. (*26*). Under vacuum, 20 mL fractions were dried, and the residues were dissolved in 6 mL 100mM carbonate buffer, pH 9.0 (albumin/globulin fraction) or 50% propanol in 100 mM carbonate buffer, pH 9.0 (gliadin and glutenin fractions). To each sample was added 3 mL 1% w/v FITC in dimethyl sulfoxide, under stirring, and labeling was performed for 2 h at room temperature. Labeled samples were dialyzed at 4 °C 24 h against the extraction buffer. Buffers were changed four times, and the first two dialysis steps contained activated charcoal. All samples were additionally separated by SEC, using a 7 × 200 mm Sepadex G-10 column (Amersham biosciences, Uppsala, Sweden), equilibrated with 20% acetonitrile in 50 mM triethylamine, and the pH was adjusted to 7.0 using CO_2. A 2.5 mL sample was diluted with 1.5 mL solvent, applied to the column, and the eluent was fractionated. Fractions containing proteins but no free FITC were pooled, lyophilized, and stored frozen. The formation of precipitates was not observed during any of the labeling and purification steps.

Strains and Culture Conditions. The sourdough isolates *Lactobacillus sanfranciscensis* LTH2581 (*27*) and *Lactobacillus pontis* TMW 1.397 (=DSM 8475^T) (*28*) were grown in modified de Man, Rogosa, Sharpe medium (*29*) containing 10 g L^{-1} maltose and 5 g L^{-1} each of glucose and fructose. To obtain solid media, 17 g L^{-1} agar-agar was added . *L. sanfranciscensis* was grown at 30 °C, *L. pontis* was grown at 37 °C, and plates were incubated anaerobically. 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 tap water, resuspended in 1 mL tap water, and used immediately to inoculate the doughs.

Reconstitution of Wheat Doughs with Fluorescent Labeled Proteins and Dough Fermentation. The wheat flour used for extraction and labeling of protein fractions was also used for dough fermentations. Six doughs with a dough yield of 225 were prepared according to the dough formulas shown in **Table 1**, mixed to homogeneity with a spatula (1 min mixing time), and incubated at 30 °C. The addition of labeled protein fractions to different dough systems was performed in all six dough systems, with addition of the three different protein fractions. The following protein additions were used: FITC−albumin/globulin, FITC−gliadin, FITC−glutenin. 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 a protease preparation to an acid control dough and a dough fermented with *L. pontis*. Samples were taken from the doughs after 0, 7, and 24 h of fermentation. Cell counts were determined as described (*7*). Dough samples were extracted immediately with 1.5% SDS for subsequent SED analysis as described below.

SEC of SDS-Soluble Proteins. Dough samples were characterized by SEC with respect to the relative amounts of SDS-soluble proteins as well as their size distribution. Dough (100 mg) was extracted with 400 μl of 1.5% SDS in 50 mM sodium phosphate buffer, pH 6.9 (*26*). After centrifugation, the supernatant was applied to a Superdex 200 column coupled to a Superdex peptide column (both columns Amersham biosciences, Uppsala, Sweden), to achieve fractionation in the molecular weight (MW) range of 100 to 5 × 10^6. Samples were eluted with 50 mM sodium phosphate buffer, pH 8.5, containing 0.1% SDS

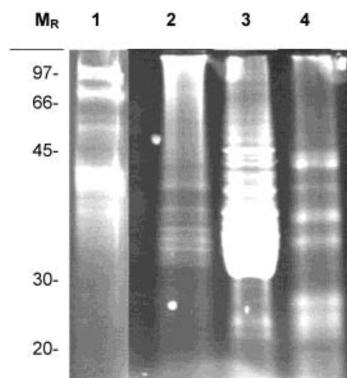

Figure 1. Separation of FITC-labeled wheat protein fractions by SDS–PAGE and detection of fluorescence. Lane 1: reduced sample of SDS-extracted polymeric glutenin after dialysis. Lanes 2, 3, and 4: nonreduced samples of SDS-extracted, polymeric glutenin, gliadin, and albumin/globulin, respectively, after gel chromatography. To the left is indicated the migration in the gel of the molecular weight markers apparent after Coomassie staining of the gel.

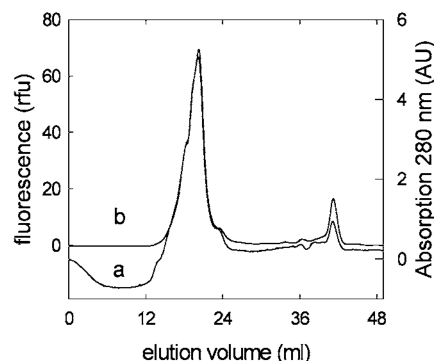

Figure 2. Separation of FITC-labeled gliadins after gel chromatography by SEC, fluorescence is compared to adsorption at 280 nm. Trace **a**: UV-280 traces specifically represent the protein content. Trace **b**: Fluorescence trace (exitation wavelength 485 nm, emission wavelength 520 nm).

and 20% acetonitrile at a flow of 0.4 mL min^{-1}. The UV detector was set to 210 and 280 nm, and the 210 nm UV trace was used to quantify proteins, unless otherwise specified. The fluorescence detector was set to excitation wavelength of 485 nm and emission wavelength of 520 nm. The columns were calibrated using the high molecular weight and low molecular weight (HMW and LMW) calibration kits (Amersham biotech, Uppsala, Sweden). Additionally, the elution volumes of polymeric and monomeric proteins and LMW compounds were determined by analysis of extracts from unfermented flour. Fractions were collected and analyzed by native and reduced SDS−polyacrylamide gel electrophoresis (PAGE) as described below.

SDS−PAGE. SDS−PAGE analysis of flour proteins were performed in a BioRad Mini-Protean 3 vertical electrophoresis cell (BioRad, Munich, Germany), using the Laemmli buffer system, according to the instructions of the manufacturer. Dough extracts and the molecular weight marker (LMW−SDS marker kit, Amersham Biosciences, Uppsala, Sweden) were mixed with 3 vol of sample buffer and heated to 90 °C for 5 min in the presence of 5% mercaptoethanol. Heating of samples in the presence of mercaptoethanol was omitted for SDS−PAGE analysis of nonreduced samples. The stacking gels contained 4% acrylamide, and the separating gel contained 12% acrylamide; both gels were 2.67% cross-linked. Proteins were visualized with Coomassie-Blue. Gels containing fluorescence labeled proteins were additionally visualized by UV transillumination and documented with a video imaging system (Herolab, Wiesloch, Germany). For silver staining, the protocol of Blum et al. (*30*) was used.

Reproducibility of the Results. Each dough fermentation was carried out at least four times with respect to cell count and SEC. Results shown are representative for quadruplicate experiments. The experimental error of extraction, chromatographic separation, and quantification of proteins was 5% or less.

RESULTS

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 G10 Sepadex. The fractions containing purified proteins were dried and analyzed by SDS−PAGE and by SEC. The fluorescence pattern of the labeled and purified protein fractions after separation by SDS−PAGE is shown in **Figure 1**. 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); therefore, it was not considered in further experiments.

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 relative molecular weight (M_r) of less than 14 000 in addition to proteins. Protein patterns of gliadin and polymeric glutenin fractions as visualized by Coomassie staining were not modified by the labeling procedure (data not shown), and no proteins with an M_r of less than 24 000 were observed in the labeled gliadin and glutenin fractions **(Figure 1)**, 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 M_r 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 1**, 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 for FITC−gliadin are shown in **Figure 2**. Fluorescence as well as UV absorption at 280 nm was 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 M_r of less than 500. A comparable result was obtained upon analysis of the labeled albumin/globulin fraction. Fluorescence of low molecular weight compounds (M_r < 500) amounted to 30% in the glutenin fraction.

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 24 h and SDS-soluble proteins were extracted and analyzed by SEC **(Figure 3)**. 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

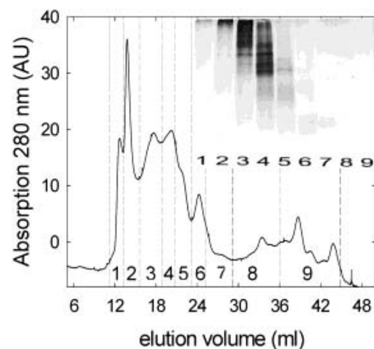

Figure 3. Fractionation of SDS soluble proteins by SEC and separation of these fractions by SDS–PAGE without reduction of the samples. UV-280 nm traces represent extraction from a neutral control dough fermented for 24 h. Different fractions are indicated with dotted lines, and the numbering of lanes in the gel corresponds to the numbering of fractions. Equal amounts of the fractions were separated by SDS–PAGE and silver stained. 12–18 mL elution volume: polymeric proteins with M_r > 200k. 18–24 mL elution volume: monomeric proteins. 24–44 mL elution volume: peptides and amino acids.

Figure 3. Lanes 1, 2, and 3 in **Figure 3** contain polymeric proteins. In lane 3 to lane 5, monomeric proteins and peptides with M_r 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 12−18 mL, monomeric proteins at 18−24 mL, and amino acids and peptides with a molecular weight of less than 5000 elute at 30−44 mL.

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

Sourdough samples were extracted immediately after dough mixing and after 24 h 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.

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 1)**. 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 (12−24 mL) and peptides/amino acids (24−40 mL) is shown in **Table 2**. The percentage values were calculated on the basis of 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 only 79%.

Table 2. Recovery of FITC–Gliadin and FITC–Glutenin as Proteins and Hydrolysis Products from Doughs Fermented for 24 Hours[a]

dough	FITC–gliadin		FITC–glutenin	
	proteins[b]	peptides	proteins	peptides
neutral control 0 h	75	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] Values are expressed as a percentage of the fluorescence chromatogram areas corresponding to proteins and peptides relative to the total fluorescence chromatogram areas of unfermented doughs. [b] 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.

In neutral control doughs, no significant increase was detectable during fermentation in peptides/amino acids when either labeled gliadines 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 gliadines **(Table 2, Figure 4A)**. No significant differences were observed between sourdoughs and acid control doughs when the sum of LMW fluorescences were compared. However, differences in size distribution of fluorescence labeled peptides in acid control doughs and sourdoughs were observed **(Figure 4B)**. Between 28 and 35 mL elution volume, the amount of fluorescence was higher in acid control doughs compared to sourdoughs. Between 35 and 40 mL, the elution volume corresponding to small peptides or amino acids and the highest fluorescence were 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 h. On the basis of the fluorescence chromatograms, the percentage of proteins and peptides/amino acids was calculated **(Table 2)**. 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*.

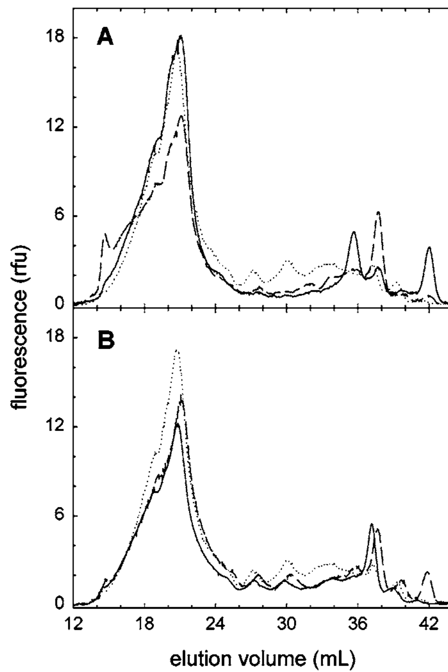

Figure 4. Separation of SDS soluble proteins from doughs containing fluorescence labeled gliadin by SEC. (A) Fluorescence trace represents extractions from neutral control dough at 0 h (solid line), extractions after 24 h of fermentation from neutral control dough (dashed line), and acid control dough (dotted line). (B) Fluorescence trace represents extractions after 24h of fermentation from sourhdough with *L. pontis* (solid line) and sourdough with *L. sanfranciscensis* (dashed line); the extraction from 24 h fermented acid control dough is furthermore included for comparison (dotted line).

Incorporation of Proteins into the Gluten Macropolymer. SEC analysis of SDS-soluble proteins allows 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 *(13, 17, 31)*. This biochemical process is dependent on enzyme catalyzed processes in wheat dough. FITC−glutenin was added to all doughs **(Table 1)** 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 5A**. 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 h, less fluorescence is SDS-extractable, and 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 $> 2 \times 10^6$) 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.

Fluorescence chromatograms of SDS-soluble proteins extracted from an acid control dough with addition of FITC−glutenin are shown in **Figure 5B**. 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.

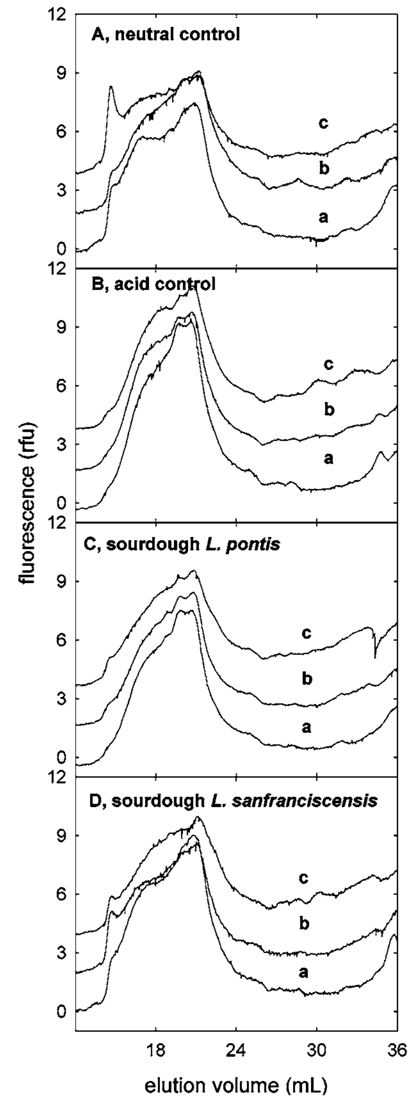

Figure 5. Separation of SDS soluble proteins from doughs containing fluorescence labeled glutenin by SEC. Fluorescence trace represents extractions from doughs extracted after 0 (**a**), 7 (**b**), and 24 (**c**) hours. Shown are chromatograms for neutral control doughs (A), acid control dough (B), sourdoughs fermented with *L. pontis* (C), and sourdoughs fermented with *L. sanfranciscensis* (D). Polymeric proteins elute at 12–18 mL, monomeric proteins at 18–24 mL, and peptides/amino acids at 24–44 mL. Chromatogram traces were cut off at 36 min to highlight shifts in HMW peaks.

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 5**, parts C and D for *L. pontis* and *L. sanfranciscensis*, respectively, after 0, 7, and 24 h fermentation. Doughs fermented with *L. pontis* and *L. sanfranciscensis* showed virtually identical chromatograms compared to neutral doughs during the first 7 h. 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 and the overall amount of fluorescence increased again to levels of unfermented doughs.

Table 3. Recovery of FITC–Gliadins as Polymeric and Monomeric Proteins from Neutral and Acid Control Doughs and Sourdoughs Fermented with *L. pontis* and *L. sanfranciscensis*[a]

dough	ratio polymeric/monomeric proteins[b]		
	0 h	7 h	24 h
neutral control	0.23	0.37	0.41
acid control	0.18	0.18	0.20
L. pontis	0.21	0.30	0.25
L. sanfranciscensis	0.23	0.38	0.22

[a] Shown are the ratios of peak areas corresponding to polymeric and monomeric fluorescent proteins. [b] Values were calculated based on the fluorescent trace of SEC-analysis. Elution volumes of 12–18 mL corresponded to polymeric proteins, elution volumes of 18–24 mL corresponded to monomeric proteins.

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**. The total fluorescence recovered from the doughs after fermentation, including the neutral dough, varied by less than 6%, indicating that incorportation 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 24 h of fermentation (**Table 3**, **Figure 4A**). In acid control doughs, there is virtually no change during fermentation time; the ratio remained stable between 0.18 and 0.20. A polymerization of FITC−gliadin at acidic conditions can therefore be ruled out. During the first 7 h of fermentation with lactobacilli, corresponding to a dough pH > 4.5, the ratio increased from about 0.22 at 0 h to 0.30 for *L. pontis*, and to 0.38 for *L. sanfranciscensis*. After 24 h 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 h 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).

DISCUSSION

Sourdough fermentations are known to affect wheat proteins, which influence dough rheology, flavor, and bread texture. In previous work, the release of amino acids (*7, 23, 32*) and the hydrolysis of proteins during sourdough fermentation (*12*) was investigated. So far, it was not possible to observe, in situ, distinct protein fractions during fermentation in food matrixes. 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 (*24*). 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 were detectable when native proteins were compared with FITC-labeled proteins by SDS−PAGE.

Wheat proteolytic enzymes 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 LMW fluorescence was observed. Several proteases from wheat endo- and exosperm have been characterized (*11*), and several of them have their maximum activity at low pH values (*10*). Degradation was observed of not only the water soluble substrates albumins and globulins but also the water insoluble gliadins and glutenins. 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 degradation of gliadin and glutenin proteins 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 (*7*). However, whereas microbial enzymes do not influence to a great extent 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 (*12*). In this study, it 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 LMW fluorescence remained unaltered.

The proteolytic system of lactic acid bacteria consists of cell wall associated proteinases, which convert proteins to oligopeptides (*33*). Di-, tri-, and oligopeptide transport is the main route for nitrogen entry into the bacterial cells,virtually all peptidases are located intracellularly (*34, 35*), and uptake of peptides is preferred over amino acid uptake (*36*). 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 (*37, 38*). The proteolytic system of *L.* sanfranciscensis compares well to that of other lactic acid bacteria (*34*). Berg et al. (*39*) isolated a peptide from yeast extract that enhances growth of *L. sanfranciscensis*. Our data suggest that lactobacilli in sourdough fermentations not only are capable of growing on peptides as the only source for essential amino acids but also prefer the uptake of peptides over amino acid transport.

The addition of a fungal protease to an 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. (*26*). 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, it was observed that 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 influenced not only by the molecular size but also by the density of the molecules (*40*). 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 (*14, 26*). 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−gliadines 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 h, 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 h, 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 matrixes not only is 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.

In conclusion, a method based on the labeling of proteins with FITC was established, which allows determination of the fate of individual proteins in food fermentations. The method was successfully applied to determine the (de-)polymerization and proteolytic degradation of gluten proteins in wheat sourdoughs. During sourdough fermentation, proteolytic degradation of gluten proteins and depolymerization of the gluten macropolymer were observed that could be mainly attributed to dough pH and cereal enzyme activity.

ABBREVIATIONS USED

GMP, glutenin macropolymer; FITC, fluorescein isothiocyanate; SEC, size exclusion chromatography; HMW, high molecular weight; LMW, low molecular weight; LTH, strain collection Lebensmittel-technologie, Universität Hohenheim, Stuttgart, Germany; TMW, strain collection Technische Mikrobiologie, TU München, Freising, Germany; DSM, strain collection German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany.

LITERATURE CITED

(1) Wolf, G. Traditional fermented food. In: Anke, T. (Ed.) Fungal Biotechnology. Chapman & Hall, Weinheim. 1997; 3−13.

(2) Yokotsuka, T., Sasaki, M. Fermented protein foods in the orient, Shoyu and Miso in Japan. In *Microbiology of fermented food*. Wood, B. J. B. Ed. Chapman & Hall: London, 2001; pp 351−415.

(3) Smit, G., Verheul, A., van Kranenburg, R., Ayad, E., Siezen, R., Engels, W. Cheese flavour development by enzymatic conversions of peptides and amino acids. *Food Res. Int.* **2000**, *33*, 153−160.

(4) Ordonez, J. A., Hierro, E. M., Bruna, J. M., de la Hoz, L. Changes in the components of dry-fermented sausages during ripening. *Crit. Rev. Food Sci. Nutr.* **1999**, *39*, 329−367.

(5) Collar Esteve, C., Benedito de Barber, C., Martinez-Anaya, M. Microbial sour doughs influence acidification properties and breadmaking potential of wheat dough. *J. Food Sci*. **1994**, *629−633*, 674.

(6) Corsetti, A., Gobbetti, M., Balestrieri, F., Paoletti, F., Ruddi, L., Rossi, J. Sourdough lactic acid bacteria effects on bread firmness and staling. *J. Food Sci*. **1998**, *63*, 347−351.

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

(8) Hansen, A., Hansen, B. Flavour of sourdough wheat bread crumb. *Z. Lebensm. Unters. Forsch.* **1996**, *202*, 244−249.

(9) Schieberle, P. Intense aroma compounds − Useful tools to monitor the influence of processing and storage on bread aroma. *Adv. Food Sci.* **1996**, *18*, 237−244.

(10) Bleukx, W., Delcour, J. A. A second aspartic proteinase associated with wheat gluten. *J. Cereal Sci*. **2000**, *32*, 31−42.

(11) Bleukx, W., Roels, S. P., Delcour, J. A. On the presence and activities of proteolytic enzymes in vital wheat gluten. *J. Cereal Sci*. **1997**, *26*, 183−193.

(12) di Cagno, R., de Angelis, M., Lavermicocca, P., de Vicenzi, M., Giovannini, C., Faccia, M., Gobbetti, M. Proteolysis by sourdough lactic acid bacteria: effects on wheat flour protein fractions and gliadin peptides involved in human cereal intolerance. *Appl. Environ. Microbiol*. **2002**, *68*, 623−633.

(13) Weegels, P. L., Hamer, R. J., Schofield, J. D. Depolymerisation and re-polymerisation of wheat glutenin during dough processing. II. Changes in composition. *J. Cereal Sci.* **1997**, *25*, 155−163.

(14) Lindsay, M. P., Skerritt, J. H. The glutenin macropolymer of wheat flour doughs: structure−function perspectives. *Trends Food Sci. Technol.* **1999**, *10*, 247−253.

(15) Grosch, W., Wieser, H. Redox reactions in wheat dough as affected by ascorbic acid. *J. Cereal Sci*. **1999**, *29*, 1−16.

(16) Tilley, K. A., Benjamin, R. E., Bagoroza, K. E., Okot-Kotber, b. M., Prakash, O., Kwen, H. Tyrosine cross-links: Molecular basis of gluten structure and function. *J. Agric. Food Chem.* **2001**, *49*, 2627−2632.

(17) Weegels, P. L., Hamer, R. J., Schofield, J. D. Functional properties of wheat glutenin. *J. Cereal Sci*. **1996**, *23*, 1−18.
(18) Sapirstein, H. D., Suchy, J. SDS−Protein gel test for prediction of bread loaf volume. *Cereal Chem.* **1999**, *76*, 164−172.
(19) Dachkevitch, T. and Autran, J.-C. Prediction of baking quality of bread wheats in breeding programs by size-exclusion high-performance liquid chromatography. *Cereal Chem*. **1989**, *66*, 448−456.
(20) Gupta, R. B., Khan, K. and MacRitchie, F. Biochemical basis of flour properties in bread wheats. I. Effects of variation in the quality and size distribution of polymeric protein. *J. Cereal Sci*. **1993**, *18*, 23−41.
(21) Wehrle, K., Arendt, E. K. Rheological changes in wheat sourdough during controlled and spontaneous fermentation. *Cereal Chem.* **1998**, *75*, 822−886.
(22) Wu, J. Y., Hoseney, R. C. Rheological changes in cracker sponges during an 18-hour fermentation. *Cereal Chem.* **1989**, *66*, 182−185.
(23) Gobbetti, M., Simonetti, M. S., Cossignani, J., Corsetti, A., Damiani, P. Free D- and L- amino acid evolution during sourdough fermentation and baking. *J. Food Sci*. **1994**, *59*, 881−884.
(24) Twining, S. S. Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes. *Anal. Biochem.* **1984**, *143*, 30−34.
(25) Kruger, J. E., Marchylo, B. A., Hatcher, D. Preliminary assessment of a sequential extraction scheme for evaluating quality by reversed-phase high performance liquid chromatography and electrophoretic analysis of gliadins and glutenins. *Cereal Chem.* **1988**, *65*, 208−214.
(26) Lindsay, M. P., Tamas, L., Appels, R., Skerritt, J. H. Direct evidence that the number and location of cysteine residues affect glutenin polymer structure. *J. Cereal Sci.* **2000**, *31*, 321−333.
(27) Böcker, G., Vogel, R. F., Hammes, W. P. *Lactobacillus sanfrancisco* als stabiles Element in einem Reinzucht-Sauerteig-Präparat. *Getreide Mehl Brot* **1990**, *44*, 269−274.
(28) Vogel, R. F., Böcker, G., Stolz, P., Ehrmann, M., Fanta, D., Ludwig, W., Pot, B., Kersters, K., Schleifer, K. H., Hammes, W. P. Identification of lactobacilli from sourdough and description of *Lactobacillus pontis* sp. nov. *Int. J. Syst. Bacteriol.* **1994**, *44*, 223−229.
(29) Stolz, P., Böcker, G., Hammes, W. P., Vogel, R. F. Utilization of electron acceptors by lactobacilli isolated from sourdough. I. *Lactobacillus sanfrancisco*. *Z. Lebensm.-Unters. Forsch. A* **1995**, *201*, 91−96.
(30) Blum, H., Beier, H., Gross, H. J. Improved silver staining of plant proteins, RNA, and DNA in polyacrylamide gels. *Electrophoresis* **1987** *8*, 93−99.
(31) Skerritt, J. H., Hac, L., Bekes, F. Depolymerization of the glutenin macropolymer during dough mixing: I. Changes in levels, molecular weight distribution, and overall composition. *Cereal Chem.* **1999**, *76*, 395−401.
(32) Collar, C., Mascaros, A. F., Benedito de Barber, C. Changes in free amino acids during fermetation of wheat doughs started with pure culture of lactic acid bacteria. *Cereal Chem*. **1991**, *68*, 66−72.
(33) Siezen, R. J. Multidomain, cell-envelope proteinases of lactic acid bacteria. *Antonie van Leeuwenhoek* **1999**, *76*, 139−155.
(34) Gobbetti, M., Smacchi, E., Corsetti, A. The proteolytic system of *Lactobacillus sanfrancisco* CB1: Purification and characterization of a proteinase, a dipeptidase, and an aminopeptidase. *Appl. Environ. Microbiol*. **1996**, *62*, 3220−3226.
(35) Kunji, E. R., Mierau, I., Hagting, A., Poolman, B., Konings, W. N. The proteolytic systems of lactic acid bacteria. *Antonie van Leeuwenhoek* **1996**, *70*, 187−221.
(36) Foucaud, C., Hemme, D., Desmazeaud, M. Peptide utilization by *Lactococcus lactis* and *Leuconostoc mesenteroides*. *Lett. Appl. Microbiol*. **2001**, *32*, 20−25.
(37) Guédon, E., Renault, P., Ehrlich, S. D., Delorme, C. Transcriptional pattern of genes coding for the proteolytic system of *Lactococcus lactis* and evidence for coordinated regulation of key enzymes by peptide supply. *J. Bacteriol.* **2001**, *183*, 3614−3622.
(38) Hebert, E. M., Raya, R. R., de Giori, G. S. Nutritional requirements and nitrogen-dependent regulation of proteinase activity of *Lactobacillus helveticus* CRL 1062. *Appl. Environ. Microbiol.* **2000**, *66*, 5316−5321.
(39) Berg, R. W., Sandine, W. E., Anderson, A. W. Identification of a growth stimulant for *Lactobacillus sanfrancisco Appl. Environ. Microbiol*. **1981**, *42*, 786−788.
(40) Carceller, J.-L., Aussenac, T. Size characterisation of glutenin polymers by HPSEC−MALLS. *J. Cereal. Sci*. **2001**, *33*, 131−142.

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