LC-MS/MS quantitation of α-amylase/trypsin inhibitor CM3 and glutathione during wheat sourdough breadmaking

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Running headline: Quantitation of ATI in sourdough bread

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Abstract

Aims: This study aimed to quantify α-amylase trypsin inhibitor (ATI) CM3 and glutathione (GSH) during wheat sourdough breadmaking.

Methods and results: Breads were made with two wheat cultivars and fermented with *Fructilactobacillus sanfranciscensis*, *F. sanfranciscensis ΔgshR* or *Latilactobacillus sakei;* chemically acidified and straight doughs served as controls. Samples were analysed after mixing, after proofing and after baking. GSH and CM3 were quantified by multi-reaction-monitoring-based methods on an LC-QTRAP mass spectrometer. Undigested ATI extracts were further examined by SDS-PAGE.

Conclusions: GSH abundance was similar after mixing and after proofing but increased after baking (P<0.001), regardless of fermentation. In breads baked with cv. Brennan, the samples fermented with lactobacilli had higher GSH abundance (P<0.001) than in the controls. CM3 relative abundance remained similar after mixing and after proofing but decreased after baking (P<0.001) across all treatments. This trend was supported by the SDS-PAGE analysis in which ATI band intensities decreased after baking (P<0.001) in all experimental conditions. The overall effect of baking exerted a greater effect on the abundances of GSH and CM3 than fermentation conditions.

Significance and Impact of the Study: This is the first report to quantify ATI over the course of breadmaking by LC-MS/MS in sourdough and straight dough processes.

Key words: LC-MS/MS; alpha-amylase/trypsin inhibitor; sourdough; glutathione; wheat allergens; Non-celiac wheat sensitivity, lactic acid bacteria, *Fructilactobacillus sanfranciscensis*

Introduction

Wheat is an important dietary staple and source of macro- and micronutrients in many cultures across the globe. However, some people experience adverse health effects after wheat consumption that prevent them from enjoying foods from wheat. These include disorders mediated by gluten proteins such as celiac disease and wheat allergy, as well as non-gluten-mediated conditions such as baker's asthma and non-celiac wheat sensitivity (NCWS) (Matsuo, Yokooji & Taogoshi, 2015; Leonard *et al.*, 2017). Baker's asthma is an asthmatic reaction to the inhalation of wheat flour, while NCWS is characterized by a variety of symptoms including abdominal discomfort, bloating, headaches, and irritable bowel syndrome-like symptoms. The pathology and triggers of NCWS are not fully understood, but the condition has been attributed to indigestible carbohydrates known as fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs) and is also possibly linked to non-gluten wheat proteins including α-amylase/trypsin inhibitors (ATI) (Schuppan *et al.*, 2015; Schuppan & Gisbert-Schuppan, 2019). The current solution for wheat-related health conditions is elimination of wheat from the diet. However, wheat-free diets are challenging to adopt due to the risk of nutrient deficiencies, moreover, and wheat-free alternatives are often more expensive and less readily available (Vici *et al.*, 2016). Novel processing technologies to remove allergens from wheat-based foods could be valuable for consumers with wheat sensitivity.

α-Amylase/trypsin inhibitors (ATI) are non-gluten storage proteins in wheat that have been suggested as triggers for NCWS (Geisslitz *et al.*, 2021). *In vivo* experiments indicate that ATI stimulate pro-inflammatory cytokine release via the TLR4 pathway (Bellinghausen *et al.*, 2018). The most studied ATI isoforms are the monomer 0.28, the homodimer 0.19 and the tetramer, which is comprised of one CM1 or CM2 residue, one CM16 or CM17 residue and two CM3 residues.

The isoforms 0.19 and CM3 are some of the most bioactive moieties (Tatham & Shewry, 2008; Zevallos *et al.*, 2017); for this reason, this study chose to focus on the latter. Similar to gluten, ATI are stabilized against thermal denaturation and proteolysis by multiple disulfide bonds. Geisslitz *et al.* (2021) provided a recent and comprehensive review on current knowledge on the structure and function of ATI, its potential role in adverse reactions to wheat and its fate during food processing. Hence, novel methods to reduce ATI content in food, including fermentation, may be beneficial to those who suffer from NCWS.

Sourdough involves the fermentation of grain with lactic acid bacteria (LAB) and yeast to produce breads with enjoyable organoleptic properties (Corsetti & Settanni, 2007; Gänzle, 2014). LAB modulate protein structure via acidification of the food matrix and via accumulation of low molecular weight thiols, such as glutathione (GSH) (Gänzle, Loponen & Gobbetti, 2008). During fermentation, lactobacilli lower the pH of the dough towards the pH optimum of endogenous wheat proteases, thereby increasing enzymatic activity and protein solubility (Gänzle *et al.*, 2008). Heterofermentative lactobacilli may also possess glutathione reductase, which reduces GSH in a paired reaction with NAD+ regeneration as a part of their central carbohydrate metabolism (Gänzle, Vermeulen & Vogel, 2007). GSH has a reactive cysteine residue that reduces intramolecular disulfide bonds in wheat proteins. These thiol exchange reactions result in depolymerization, increased solubility, and exposure of cleavage sites to proteolytic enzymes (Joye, Lagrain & Delcour, 2009). Redox agents and sourdough fermentation thus impact gluten structure gluten structure (Grosch & Wieser, 1999; Vermeulen *et al.*, 2006; Di Cagno *et al.*, 2010). However, whether or not sourdough exerts a similar effect on non-gluten wheat proteins including ATI is unknown.

LC-MS is an accurate and specific analytical method for identification and quantitation of food allergens (Monaci & Visconti, 2009). Several studies have developed LC-MS/MS methods to quantify ATI in wheat flour and related grains, indicating that ATI content varies between cultivars, environment and grain type (Prandi *et al.*, 2013; Geisslitz *et al.*, 2018). The effect of lactic acid bacteria on ATI bioactivity was also studied. ATI-degrading lactic acid bacteria were reported to decrease the pro-inflammatory immune reaction in mice when supplemented as probiotics (Caminero *et al.*, 2019). Sourdough fermentation also reduced ATI tetramers to monomers and reduced the cytokine release in human monocytic cells in response to sourdough extracts (Huang *et al.*, 2020). Until now, the use of LC-MS/MS methods to quantify ATI during breadmaking has not been reported. In addition, this study aimed to understand whether the abundance of GSH relates to the abundance of ATI during sourdough breadmaking. Very few studies, however, have quantified GSH by LC-MS/MS in the context of wheat or bread systems (Chen & Schofield, 1996; Reinbold *et al.*, 2008; Tang, Zhao & Gänzle, 2017; Xu *et al.*, 2018). Therefore, LC-MS/MS methods for the quantification of ATI CM3 and GSH were applied at various stages of sourdough breadmaking. The results of such analyses may help elucidate how food processing affects the allergen content of wheat-based foods.

Materials and Methods

Wheat flour

Sourdough bread was baked using two pure wheat cultivars: Red Fife and Brennan. The cultivars were stored at −20 °C and ground into flour using a 0.5 mm screen on a Retsch ZM200 ultra centrifugal mill (Retsch, Germany). Commercial whole wheat flour was used for general method development.

Reagents and standard solutions

Iodoacetamide (IAM), HPLC grade formic acid, dithiothreitol (DTT), γ-L-glutathione (GSH), DL-lactic acid 90% and acetic acid glacial 99.7% were obtained from Sigma Aldrich (Oakville, ON, Canada). Ammonium bicarbonate, LC-MS grade water and LC-MS grade acetonitrile were supplied by Fisher Scientific (Ottawa, ON, Canada). Deuterated glutathione (d_5-GSH) was purchased from SantaCruz Biotechnology (Dallas, TX, USA). Trypsin Gold Mass Spectrometry Grade and ProteaseMAX Surfactant Trypsin Enhancer were obtained from Promega (Madison,WI, USA). Two custom, isotope labelled peptides corresponding to select CM3 peptides were synthesized by BaChem (Torrance, CA, USA): (1) H-Tyr-Phe-Ile-Ala-[^{13}C6;^{15}N]Leu-Pro-Val-Pro-Ser-Gln-Pro-Val-Asp-Pro-Arg-OH; (2) H-Ser-Gly-Asn-Val-Gly-Glu-Ser-Gly-[^{13}C6;^{15}N]Leu-Ile-Asp-Leu-Pro-Gly-Cys-Pro-Arg-OH. Standard solutions of GSH and d_5-GSH were dissolved in 50% acetonitrile and alkylated with IAM. Standard solutions for the CM3 peptides were dissolved in 1% acetic acid, diluted with 0.1% formic acid, and alkylated with IAM. Mini-PROTEAN TGX Precast Protein Gels (4-20%) were obtained from BioRad.

Bacterial strains and culture conditions

Three strains of lactic acid bacteria were used, *Fructilactobacillus sanfranciscensis* DSM20451, (2) isogenic mutant *Fructilactobacillus sanfranciscensis* DSM2045118Δ*gshR* which lacks the enzyme glutathione reductase, and *Latilactobacillus sakei* TMW1.22 (Vermeulen *et al.*, 2006; Jänsch *et al.*, 2007). Strains were grown in modified de Man, Rogosa and Sharpe (mMRS) media with the carbohydrates autoclaved separately to limit Maillard reactions (Tovar & Gänzle, 2021). Cultures were grown at 30 °C without agitation.

Sourdough fermentation

Lactobacilli were streaked on mMRS agar, and incubated for 24-48 h, and single colonies were subcultured in mMRS broth. After incubation at 30 °C for 24 h, cells were washed twice with sterile tap water, resuspended in 10 mL of sterile tap water and used to inoculate 10 g of wheat flour. Each inoculum was incubated at 30 °C for 24 h. This first sourdough was mixed with 20 g wheat flour and 20 mL tap water and incubated at 32 °C for 2.5 h. Bread dough was prepared by mixing the stage two sourdough with 70 g wheat flour, 30 mL tap water, 2 g salt, 2 g sugar and 0.5 g active dry yeast. The dough was mixed for 6 min, rested for 1 h at 32 °C, shaped and then proofed for 1 h at 32 °C and baked in a forced convection oven for 12 min at 190 °C. Chemically acidified controls were acidified to pH 3.5±2 using a solution of acetic and lactic acid at a ratio of 1:4 and prepared identically to the sourdough bread. Straight dough controls were made by mixing 100 g of wheat flour with 55 mL tap water, 2 g salt, 2 g sugar and 0.5 g active dry yeast; all other steps were identical to the sourdough bread protocol. After baking, the breads were cooled before being sampled. Samples were collected from several different time points: from the flour, after mixing, after proofing and after baking (Tang *et al.*, 2017).

Determination of cell counts and pH

To determine pH and cell counts of each sourdough, 1 g of each sample was dissolved in 9 mL of deionized water and analysed using a pH meter. For cell counts, one gram of sample was dissolved in 9 mL of sterile deionized water and serial dilutions were made by diluting 100 µL of sample in 900 µL of sterile deionized water. A Whitley Automatic Spiral Platter (Don Whitley Scientific, England) was used to plate the dilutions. The plates were incubated for 24-48 h at 30 °C before being counted.

Glutathione extraction and sample preparation

Flour and bread samples (100 mg and 200 mg, respectively) were extracted in 500 µL of 80% ethanol (EtOH). Samples were alkylated with iodoacetamide (IAM): the pH was adjusted to 8 using NH_4OH, samples were mixed with 10 µL 200 mM IAM and left in the dark for 30 min at room temperature, after which the pH was lowered to 4 with formic acid. Samples were sonicated for 10 min, centrifuged at 4 °C for 2 min and the supernatant was collected. This extraction was repeated, the supernatants were combined and then centrifuged for 15 min at 4 °C. The resulting supernatant was collected, filtered with 0.2 µm filter and diluted with 50% acetonitrile and the internal standard (Reinbold *et al.*, 2008; Xiong *et al.*, 2009; Tang *et al.*, 2017).

LC-MS/MS quantitation of glutathione

Glutathione was quantified by LC-MS/MS using a targeted multiple reaction monitoring (MRM) approach. Samples and standards were using an Agilent 1200 series HPLC system coupled to a 3200 QTRAP mass spectrometer (SCIEX) with a TurboIonSpray source operating in positive ion mode. Eluant A was 0.1% formic acid in acetonitrile while Eluant B was 0.1% formic acid in water. For the separation on an Ascentis Express HILIC column (10 cm x 2.1 mm x 2.7 µm), eluant A was 0.1% formic acid in acetonitrile while B was 0.1% formic acid in water. The following gradient was used: 0-10 min, 95-80% A; 10-10.1 min, 80-95% A; 10.1-20 min, 95% A. Data acquisition and peak integration was carried out using Analyst 2.0 (SCIEX, Redwood City, CA, USA). The MRM transitions that were selected for the analyte and internal standard are reported in Table 1. GSH standards were diluted in 50% acetonitrile and used to make a five-point external calibration curve from 1 ppb to 50 ppb (Figure S1).

Glutathione LC-MS/MS method validation

Sourdough made with commercial wheat and fermented with *F. sanfranciscensis* DSM20451 for 24 h at 30 °C was used for method validation. The precision of the extraction was calculated by extraction and analysis of 3 samples taken from the same wheat sourdough. This indicated good overall precision, with a measured coefficient of variance of 3%.

Recovery was determined by spiking sourdough samples before and after extraction with 100 µL of at 1 ppm, 2.5 ppm and 5 ppm GSH solutions. The GSH concentration was calculated with the calibration curve shown in Figure S1. Samples that were not spiked with standards were spiked with an equivalent amount of solvent (50% acetonitrile) to maintain identical extraction conditions. Recovery was calculated using the following equation: $[(A-C)/(B-C)]\times 100$, where A = peak area of IAM-GSH in sourdough sample spiked before extraction; B = peak area of IAM-GSH in sourdough sample spiked after extraction; C = peak area of IAM-GSH in sourdough sample not spiked with standards. The average recovery was 80%.

The limit of detection (LOD) was defined as 3 times the S/N, while limit of quantification (LOQ) was defined as 10 times the S/N. Both were determined using alkylated glutathione standards dissolved in 50% acetonitrile. The LOD was found to be 0.2 ppb and the LOQ was 1 ppb.

Identification of CM3 marker peptides

The amino acid sequence for CM3 was obtained from the literature and isolated CM3 obtained from Huang et al. was used for method development and MS optimization (García-Maroto *et al.*, 1990; Huang *et al.*, 2020). *In silico* tools from prospector.ucsf.edu were used to predict and select CM3 marker peptides: MS-digest was used to generate a list of possible peptides generated by tryptic digestion; MS-isotope was used to predict their isotope distributions; and MS-product

helped predict their MS/MS spectra. LC-QTOF was used to confirm the presence and identity of CM3 peptides in sourdough samples. The two most abundant peptides, YFIALPVPSQPVDPR and SGNVGESGLIDLPGCPR, were selected as markers for quantitation (Geisslitz *et al.*, 2018).

Extraction and digestion of CM3

Flour and bread dough samples (50 g and 100 g, respectively) were weighed out and reconstituted in 500 mL of 50 mM ammonium bicarbonate. Samples were alkylated with 10 µL of 200 mM IAM and left in the dark for 30 min. They were then centrifuged for 25 min at room temperature before the supernatant was collected. This extraction was repeated, and the supernatants were combined, centrifuged for 15 min at room temperature. The resulting the supernatant was collected and digested with MS grade trypsin in preparation for mass spectrometric analysis: samples were mixed with 44 µL of 0.1% ProteaseMAXsurfactant enhancer and reduced with 100 µL of 100 mM DTT for 30 min a 30 °C. The reduced proteins were alkylated with 10 µL of 200 mM IAM for 30 min in the dark and mixed with 1 µL 1% proteaseMAX surfactant enhancer. Samples were digested with 10µL 0.5 ug/uL Trypsin Gold at 37 °C for 1 h. To halt further reactions, the pH was lowered to < 4 with formic acid (Geisslitz *et al.*, 2018).

LC-MS/MS quantitation of ATI CM3

The CM3 tryptic digest was analysed by LC-MS/MS as described above for glutathione, but using the two transitions and their labelled internal standards with the optimized collision energies given in Table 1. Chromatographic separation used an ACE 3 AQ column (150 mm x 2.1 mm x 3 µm) with eluant A of 0.1% formic acid in acetonitrile and eluant B was of 0.1% formic acid in water. The gradient used was: 0-6 min, 0% A; 6-20 min, 100% A;20-30 min, 100% A; 30-40 min, 0% A. External calibration standards using the isotope-labelled standards were diluted with 0.1% formic

acid for a 5 point calibration curve (not shown) to ensure instrument response was linear in the range studied.

CM3 LC-MS method validation

Method precision was determined by analysis of three samples extracted from the same wheat sourdough. The sourdough was made with commercial wheat and fermented with *F. sanfranciscensis* DSM20451 for 24 h at 30 °C. An overall coefficient of variation of 8.1% was achieved.

Recovery was determined by spiking sourdough samples before and after extraction with the CM3 obtained from (Huang *et al.*, 2020) that was diluted with 50% acetonitrile 1:50, 1:20 and 1:10. Samples that were not spiked with the standard were spiked with an equivalent amount of solvent to maintain identical extraction conditions. The commercial wheat sourdough samples used in the recovery experiments were fermented with *F. sanfranciscensis DSM20451* for 24 h at 30 °C. Recovery was calculated using the following equation: $[(A-C)/(B-C)]\times 100$, where A = peak area of CM3 in sample spiked before extraction; B = peak area of CM3 in sample spiked after extraction; C = peak area of CM3 in sample not spiked with any standards. The average percent recovery was 107%.

The limit of detection (LOD), defined as 3 times the S/N, was 50 ppb for SGNVGESG[$^{13}C_6$,^{15}N]LIDLPGCPR and 100 ppb for YFIA[$^{13}C_6$,^{15}N]LPVPSQPVDPR; the limit of quantification (LOQ), defined as 10 times the S/N, for the two peptides was 75 and 100 ppb, respectively. Both were determined using alkylated, isotope labelled CM3 marker peptide standards in 0.1% formic acid.

Non-reducing SDS-PAGE analysis of ATI

Flour and bread samples (60 g flour and 100 g, respectively) were weighed out and extracted with 50 mM ammonium bicarbonate, as described above, but not digested with trypsin. Samples were mixed with 4x loading sample buffer, stained with Coomassie Blue, and separated on a 4-20% Mini-PROTEAN TGX Precast Protein Gels, 15 well, 15 µL (BioRad, Mississauga, Ontario, Canada). Thermo Scientific PageRuler Plus Prestained Protein ladder, 10 to 250 kDa, (Fisher Scientific, Ottawa, Ontario, Canada) was used for molecular markers. Running conditions were 150 V for 50 min. Protein band intensities were measured using a BioRad ChemiDoc MP Imager (BioRad, Mississauga, Ontario, Canada). The relative band intensity was calculated by normalizing the sample band intensity to that of the standard and averaging the results of three biological replicates. A sample gel is shown in Figure S2.

Statistics

Two-way ANOVAs and Tukey's HSD test were performed using R (version 4.0.5). Statistical significance was assessed at an error probability of 5% ($P<0.05$).

Results

Fermentation

Sourdough bread was fermented with three different lactobacilli. The pH and cell counts reported in Table 2 were used to verify that fermentation properly occurred. As expected, the pH of the ranged from 3.5 to 3.7 and the cell counts were in the range of 10^8, indicating acceptable growth. The colony morphologies on agar plates from samples matched the colony morphologies of the inocula. The pH of the breads ranged from 5.5 to 6.5.

Glutathione quantitation

GSH abundance was measured in wheat flour and during sourdough breadmaking by LC-MS/MS. In cv. Red Fife and cv. Brennan flour, the concentration of GSH was 35 and 56 nmol/g flour, respectively. GSH abundance in the dough during breadmaking is displayed in Figure 1. In doughs made with cv. Red Fife, GSH decreased to around 4 nmol/g after mixing, remained around 3 nmol/g after proofing and increased significantly to about 11 nmol/g after baking across all treatment types. The doughs made with cv. Brennan displayed a similar trajectory. The GSH content dropped to roughly 4 nmol/g after mixing, remained around 4 nmol/g after proofing and rose significantly to about 17 nmol/g, regardless of fermentation. GSH content was significantly higher in doughs fermented with lactobacilli than in the controls in samples made with cv. Brennan, but not in samples made with cv. Red Fife.

ATI quantitation

LC-MS/MS methods were developed and applied to measure the abundance of ATI CM3 during sourdough breadmaking (Figure 2). The relative abundance of CM3 was similar after proofing and decreased significantly after baking in all doughs made with both cv. Red Fife and cv. Brennan, regardless of fermentation type. Differences between treatments were only observed in cv. Brennan samples. The CM3 abundance in samples fermented with *L. sanfranciscensis* ΔgshR was higher than both of the controls and the CM3 abundance in samples fermented with *F. sanfranciscensis* was greater than the straight dough control.

SDS-PAGE analysis of tetrameric and monomeric isoforms

Undigested ATI sourdough extracts were also examined by SDS-PAGE to explore how fermentation might affect the proportion of tetrameric, dimeric, and monomeric ATI species

(Figure 3 and Table S1). The band intensities of both tetrameric and monomeric isoforms were significantly reduced after baking. This corroborates the LC-MS/MS data in that overall baking was the greatest determinant of ATI band intensity.

Discussion

Sourdough is applied in baking as a leavening agent or to improve bread quality. In breadmaking, the amount of inoculum used for fermentation typically ranges from less than 10 to more than 30% and our baking process was designed to mimic conditions used in industry. Fermentation length determines what aspect of the process is emphasized. While longer fermentation times, which correspond to low dosage of sourdough into the bread dough, favor acidification and protein modification by proteolysis while the shorter fermentation length used in this study correspond to higher dosages of sourdough into the bread dough and emphasize microbial metabolic activity in bread dough and protein modification by thiol-exchange reactions (Gänzle, 2014; Arora *et al.*, 2021).

The present study explored how sourdough affects bioactive wheat proteins and peptides using LC-MS/MS. The abundance of GSH in wheat flour varies widely between cultivars and due to nutrient availability. Sarwin et al. reported 16-41 nmol GSH/g and Schofield and Chen reported 18-81 nmol GSH/g across a range of pure wheat cultivars (Sarwin *et al.*, 1992; Schofield & Chen, 1995; Chen & Schofield, 1996). In wheat cv. Star, Reinbold et al. observed 84-135 nmol GSH/g flour and noted that GSH abundance is influenced by sulfur availability (Reinbold *et al.*, 2008). Our study reported 35 and 55 nmol GSH/g, which is comparable with the current literature. Over the course of a 24 h fermentation of commercial flour with lactobacilli, GSH concentrations ranged from around 6-24 nmol/g, similar to the values reported in our study for the samples after mixing (Tang *et al.*, 2017). In all treatments, GSH abundance remained similar between after mixing and

after proofing but increased significantly after baking. This suggests that the overall baking process was the most significant determinant of GSH abundance. This is supported by a previous study in which free thiol content was not found to differ significantly between mixing and proofing (Xu *et al.*, 2018). GSH content was higher in doughs fermented with lactobacilli than in controls in samples made with cv. Brennan, but it did not differ between sourdoughs. This suggests a mechanism other than glutathione reductase influences GSH abundance.

In the present study, the fate of ATI was assessed by quantification of two peptides of the CM3 subunit of tetrameric ATI. Tetrameric ATI which includes two CM3 subunits is the most abundant ATI in wheat (Geisslitz *et al.*, 2021); moreover, the tetrameric ATI including CM3 was reported to have a higher pro-inflammatory activity when compared to monomers (Zevallos *et al.*, 2017). The relative abundance of CM3 decreased significantly after baking regardless of fermentation, suggesting baking exerted a greater effect than fermentation by lactobacilli. The degradation of ATI *in vitro* (Fraberger *et al.*, 2020) as well as a reduced bioactivity of ATI in mice (Caminero *et al.*, 2019) was attributed to proteolytic activity of lactobacilli, however, sourdough lactobacilli in general and specifically the strains used in this study lack extracellular proteases and degrade peptides but not high molecular weight proteins (Vermeulen *et al.*, 2005; Gänzle *et al.*, 2008; Zheng *et al.*, 2015; Tovar & Gänzle, 2021). ATI tetramers were reduced to monomers during sourdough fermentation and this conversion was attributed to pH-dependent proteolysis but not to thiol accumulation; however, changes in ATI that were observed in different sourdoughs were no longer apparent in bread after baking (Huang *et al.*, 2020). Moreover, while sourdough may reduce tetrameric ATI to monomeric forms, the latter remain bioactive. Our study suggests that any changes to ATI that may occur during sourdough fermentation did not impact the abundance of the CM3 in bread dough or bread relative to straight dough or acidified controls. Other studies

have also reported reduction in ATI bioactivity and enzyme inhibition in baked straight bread supporting the idea that ATI abundance is diminished during the baking process (Simonato *et al.*, 2001; Kostekli & Karakaya, 2017). It is important to note that food processing and heat treatment affect the solubility and extractability of wheat proteins and the protocol for CM3 extraction employed in the present study did not account for proteins that were rendered insoluble due to protein aggregation or covalent cross-linking with other dough components during baking.

Figure 4 illustrates the mechanisms that potentially affect the structure and abundance of proteins during sourdough breadmaking. Native protein structure can be altered by acidification and pH-dependent proteolysis by wheat enzymes, thiol-mediated redox reactions, and heat denaturation. Acidification plays an important role in gluten degradation (Thiele, Gänzle & Vogel, 2003; Thiele, Grassl & Gänzle, 2004; Loponen *et al.*, 2004), but the lack of difference between chemically acidified and straight doughs in our study indicate that acidity had little effect on CM3 abundance in bread dough and bread. Redox agents modulate gluten polymerization via disulfide exchange reactions (Grosch & Wieser, 1999; Navrot *et al.*, 2018) and heterofermentative and homofermentative lactobacilli exert opposing effects on gluten proteins, attributed to differences in thiol metabolism (Vermeulen *et al.*, 2006). However, the abundance of CM3 or GSH between *F. sanfranciscensis* and its *ΔgshR* mutant did not differ, confirming prior observations that microbial glutathione reductase does not significantly impact the fate of ATI in wheat baking (Huang *et al.*, 2020). Similarly, we observed no significant differences between breads fermented with the heterofermentative *F. sanfranciscensis* and the homofermentative *L. sakei*.

In conclusion, our study demonstrated that the content of extractable ATI decreases substantially after baking. Experiments described in this study were not designed, however, to determine whether a decrease after baking relates to proteolysis in the initial stages of the baking process,

aggregation and denaturation of ATI during baking, which may reduce the extraction in absence of chaotrophic salts, or thiol-mediated linkages to other proteins. Whether the decrease in ATI concentration, or a decrease in extractability in absence of chaotrophic salts and reducing agents impacts its biological activity in NCWS thus remains subject to future investigations. Our results emphasize, however, that studies that aim to determine the impact of fermentation processes in breadmaking on the quantity and biological activity of ATI should consider the baking process as a key determinants of ATI in bread.

Acknowledgements

The Alberta Wheat Commission, the Saskatchewan Wheat Development Commission, the Minnesota Wheat Research and Promotion Council (grant No. 2018F031R), and the Natural Sciences and Engineering Research Council of Canada (NSERC, grant no. CRDPJ542616-19) are acknowledged for funding. Michael Gänzle acknowledges the Canada Research Chairs program for financial support.

Conflict of interest.

The authors declare no conflict of interest.

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Figure legends

Figure 1. LC-MS/MS quantitation of GSH during sourdough breadmaking. Error bars represent the standard deviation and n = 3, except for *n = 2. The lowercase letters indicate significant differences within treatments and the uppercase letters indicate differences between treatments, both at $P<0.01$. **Panel A.** Doughs made with cv. Red Fife. **Panel B.** Doughs made with cv. Brennan. Bar graph key: light grey = after mixing; dark grey = after proofing*; black = after baking. Abbreviations: SF = *F. sanfranciscensis*; SFΔ*gshR* = *F. sanfranciscensis*Δ*gshR*; LSAK = *L. sakei*; CA = chemically acidified dough; STR = straight dough.

Figure 2. Relative abundance of CM3 during sourdough breadmaking, as determined by LC-MS/MS. The error bars represent the standard deviation and n = 3, except for *n = 2. The lowercase letters represent differences within treatment and the uppercase letters indicate differences between treatments, both at P<0.01. **Panel A.** Doughs made with cv. Red Fife; **Panel B.** Doughs made with cv. Brennan. Bar graph key: light grey = after mixing; dark grey = after proofing*; black = after baking. Abbreviations: SF = *F. sanfranciscensis*; SFΔ*gshR* = *F. sanfranciscensis* Δ*gshR*; LSAK = *L. sakei*; CA = chemically acidified dough; STR = straight dough.

Figure 3. Relative band intensity of ATI proteins separated by SDS-PAGE during sourdough breadmaking. The error bars represent the standard deviation, and the letters indicate significant differences within treatments at P<0.01. **Panel A.** Doughs made with cv. Red Fife; **Panel B.** Doughs made with cv. Brennan. Bar graph key: light grey = ATI tetramers, dark grey = ATI monomers; no lines = after mixing, diagonal lines = after proofing*, horizontal lines = after baking. Abbreviations: SF = *F. sanfranciscensis*; SF *ΔgshR* = *F. sanfranciscensis* ΔgshR; LSAK = *L. sakei*; CA = chemically acidified dough; STR = straight dough; RFU = relative fluorescence units.

Figure 4. Schematic representation of the degradation or modification of proteins during sourdough breadmaking.

Table 1. Mass transitions and collision energies (CE) optimized for MRM detection of alkylated glutathione (IAM-GSH) and d_5-glutathione (IAM-d_5-GSH) and of CM3 marker peptides and their isotope-labelled internal standards.

Analyte / amino acid sequence	**Transition 1 (m/z)**	**CE**	**Transition 2 (m/z)**	**CE**
IAM-GSH	365.1/236.0	17	365.1/290.0	19
IAM-d_5-GSH	370.1/236.0	25	370.1/295.0	25
SGNVGESGLIDLPGCPR	864.5/586.3	55	864.5/699.3	55
SGNVGESG[$^{13}C_6$,^{15}N]LIDLPGCPR	867.9/586.3	50	867.9/699.3	50
YFIALPVPSQPVDPR	849.9/495.2	36	849.9/272.1	36
YFIA[$^{13}C_6$,^{15}N]LPVPSQPVDPR	853.0/495.2	40	853.0/272.1	40

Table 2. pH values and cell counts of sourdough, and pH values of bread. Data are shown as means ± of three independent baking trials

Flour	Fermentation	Average dough pH	Average bread pH	Average CFU/g
Red fife	*F. sanfranciscensis*	3.51 ± 0.15	5.48 ± 0.06	7.10×10^8
	F. sanfranciscensis ΔgshR	3.72 ± 0.28	5.67 ± 0.38	1.70×10^8
	L. sakei	3.45 ± 0.11	5.54 ± 0.19	6.70×10^7
	Chemically acidified	3.70 ± 0.03	5.93 ± 0.44	n/a
	Straight dough	n/a	6.25 ± 6.25	n/a
Brennan	*F. sanfranciscensis*	3.37 ± 0.03	5.82 ± 0.31	3.63×10^8
	F. sanfranciscensis ΔgshR	3.47 ± 0.01	5.87 ± 0.38	2.47×10^8
	L. sakei	3.47 ± 0.07	5.87 ± 0.43	1.12×10^8
	Chemically acidified	3.56 ± 0.03	6.27 ± 0.33	n/a
	Straight dough	n/a	6.61 ± 0.44	n/a

Online Supporting material for

LC-MS/MS quantitation of immune-reactive alpha-amylase/trypsin inhibitor CM3 and glutathione during wheat sourdough breadmaking

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Table S1. Average band intensity (RFU) and standard deviation of ATI proteins separated by SDS-PAGE during breadmaking.

Figure S1. Calibration curve for IAM-GSH.

Figure S2. SDS-PAGE separation of ATI extract from sourdough during breadmaking.

Table S1. Average band intensity (RFU) and standard deviation of ATI proteins separated by SDS-PAGE during breadmaking. N = 3 biological replicates, except *n = 2.

Flour	Sample time	Fermentation	Tetramers	Monomers
Red Fife	After mixing	*F. sanfranciscensis*	0.43 ± 0.04	0.34 ± 0.12
		F. sanfranciscensis ΔgshR	0.49 ± 0.15	0.28 ± 0.08
		L. sakei	0.50 ± 0.20	0.32 ± 0.09
		Chemically acidified	0.49 ± 0.04	0.32 ± 0.11
		Straight dough	0.50 ± 0.06	0.31 ± 0.08
	After proofing	*F. sanfranciscensis**	0.48 ± 0.09	0.29 ± 0.06
		*F. sanfranciscensis ΔgshR**	0.43 ± 0.003	0.24 ± 0.03
		*L. sakei**	0.45 ± 0.01	0.23 ± 0.01
		Chemically acidified*	0.49 ± 0.04	0.26 ± 0.06
		Straight dough*	0.44 ± 0.05	0.25 ± 0.04
	After baking	*F. sanfranciscensis*	0.12 ± 0.02	0.12 ± 0.05
		F. sanfranciscensis ΔgshR	0.12 ± 0.03	0.11 ± 0.04
		L. sakei	0.12 ± 0.02	0.13 ± 0.04
		Chemically acidified	0.16 ± 0.01	0.14 ± 0.018
		Straight dough	0.14 ± 0.04	0.14 ± 0.05
Brennan	After mixing	*F. sanfranciscensis*	0.41 ± 0.10	0.23 ± 0.04
		F. sanfranciscensis ΔgshR	0.45 ± 0.04	0.23 ± 0.06
		L. sakei	0.42 ± 0.04	0.25 ± 0.08
		Chemically acidified	0.46 ± 0.12	0.21 ± 0.03
		Straight dough	0.40 ± 0.0713	0.27 ± 0.06
	After proofing	*F. sanfranciscensis*	0.36 ± 0.03	0.24 ± 0.02
		F. sanfranciscensis ΔgshR	0.39 ± 0.003	0.26 ± 0.01
		L. sakei	0.37 ± 0.02	0.29 ± 0.02
		Chemically acidified	0.45 ± 0.06	0.27 ± 0.02
		Straight dough	0.46 ± 0.01	0.28 ± 0.03
	After baking	*F. sanfranciscensis*	0.23 ± 0.04	0.19 ± 0.02
		F. sanfranciscensis ΔgshR	0.22 ± 0.01	0.18 ± 0.02
		L. sakei	0.22 ± 0.04	0.19 ± 0.03
		Chemically acidified	0.20 ± 0.01	0.18 ± 0.04
		Straight dough	0.25 ± 0.11	0.17 ± 0.05

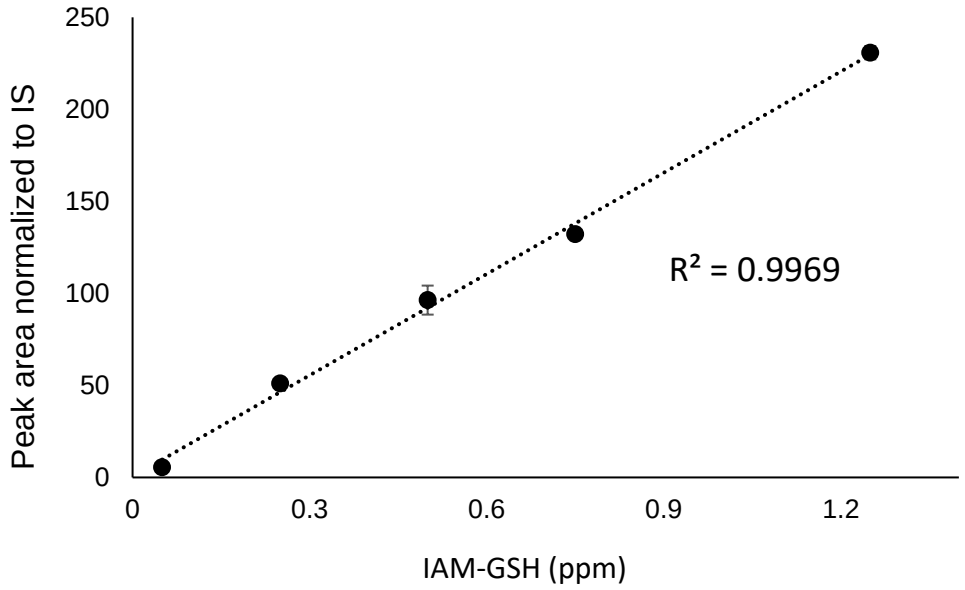

Figure S1. Calibration curve for IAM-GSH. Error bars represent the standard deviation and n = 3.

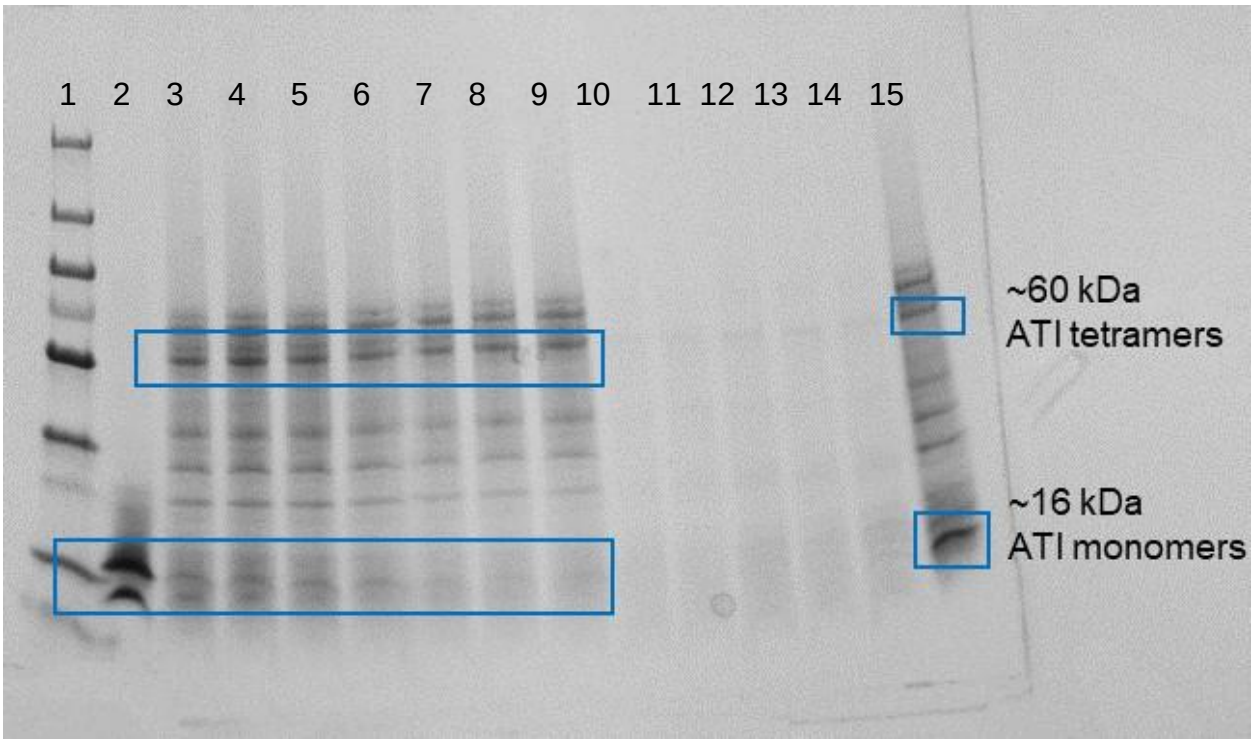

Figure S2. SDS-PAGE separation of ATI extracts from sourdoughs. All samples shown were extracted from doughs or bread prepared from the wheat cultivar Red Fife. Lanes from left to right: (1) protein ladder, (2) extracted ATI-CM3, (3) chemically acidified, after mixing; (4) straight dough, after mixing; (5) *F. sanfranciscensis* after proofing; (6) *F. sanfranciscensis ΔgshR* after proofing; (7) *Ls. sakei* after proofing; (8) chemically acidified after proofing; (9) straight dough after proofing; (10) *F. sanfranciscensis* after baking; (11) *F. sanfranciscensis ΔgshR* after baking; (12), *Ls. sakei* after baking; (13), chemically acidified after baking; (14), straight dough after baking, (15), *F. sanfranciscensis* after mixing.