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Impact of aqualysin 1 peptidase from *Thermus aquaticus* on molecular scale changes in the wheat gluten network during bread baking

Annelien E. VERBAUWHEDE¹, Marlies A. LAMBRECHT^{*1}, Ellen FIERENS^{1,1}, Oksana SHEGAY²,
Kristof BRIJS¹ and Jan A. DELCOUR¹

¹ KU Leuven, Laboratory of Food Chemistry and Biochemistry, Leuven Food Science and
Nutrition Research Centre (LForCe), Kasteelpark Arenberg 20, B-3001 Leuven, Belgium.

² Competence Center for Fermentation, Puratos Group, Rue Bourrie 12, B-5300 Andenne,
Belgium.

¹ Present address: Flanders' Food, Wetenschapsstraat 14A, B-1040 Brussel, Belgium.

^{*}Corresponding author. Phone: (+32)-16-376408. Fax: (+32)-16-321997.

E-mail: marlies.lambrecht@kuleuven.be

E-mail addresses other authors : annelien.verbauwhede@kuleuven.be;

ellen.fierens@flandersfood.com; oshegay@puratos.com; kristof.brijs@kuleuven.be;

jan.delcour@kuleuven.be

Abstract

The impact of Aqualysin 1 (Aq1), the thermo-active peptidase of *Thermus aquaticus*, on wheat albumin, globulin, gliadin and glutenin proteins during heat treatment of wheat dough and bread baking was examined. The level of protein extractable in sodium dodecyl sulfate containing medium under non-reducing conditions (SDS-EP-NR) from wheat dough decreases upon heating to a lesser extent when Aq1 is used than in control experiments. The higher SDS-EP-NR level is caused by the release by Aq1 of peptides from the repetitive gluten protein domains during baking. These peptides are also extractable from bread crumb with salt solution. The resultant thermoset gluten network in bread crumb is mainly made up by protein from non-repetitive gluten domains.

Keywords

Heat-inactivated inhibition; Covalently heat-induced protein network; Interconnected gluten peptides; Osborne type fractionation bread crumb; Sodium dodecyl sulfate extractability; Molecular weight distribution; Amino acid analysis

1. Introduction

Peptidases can be used in bread making to soften bread crumb and delay its firming. However, their activity during mixing and fermentation can impair gas retention by dough and result in open bread crumb structures and small bread volumes (Caballero, Gomez, & Rosell, 2007; Indrani, Prabhasankar, & Venkateswara Rao, 2003; Mathewson, 2000). Verbauwhe et al. (2018) applied the thermo-active serine peptidase aqualysin 1 (Aq1) from *Thermus aquaticus* in bread making. Due to its specific mode of inhibition by proteins endogenous to wheat flour

at low temperature it is inactive during dough mixing and fermentation but hydrolyzes wheat proteins at temperatures exceeding 80 °C. The latter decreases their molecular weights (MWs) and increases their extractability in sodium dodecyl sulfate (SDS) containing medium. Aq1 use has no impact on bread loaf volume but decreases the coherence of its gluten network. It further causes rather small but still significant decreases in bread crumb hardness, cohesiveness, springiness, resilience and chewiness. The large discrepancy between the impact of Aq1 on the molecular structure of wheat proteins in general and bread crumb texture in particular suggests a major role for starch in bringing about the latter (Verbauwhede, Lambrecht, Fierens, Hermans, Shegay, Brijs, et al., 2018). Nonetheless, the specificity of Aq1 towards different wheat proteins and the impact thereof on the molecular structure of the covalent heat-induced gluten network remains unknown. Further research can reveal to which extent gluten impacts the structure of wheat bread.

Wheat proteins can be divided in albumin (about 14%), globulin (about 7%), gliadin (about 33%) and glutenin (about 46%) (Belitz, Grosch, & Schieberle, 2009) by sequential extraction of wheat flour with water, salt, ethanol and acid or alkaline solutions, respectively (Osborne, 1907). Gliadin and glutenin together are referred to as gluten.

The main wheat albumin and globulin have MWs lower than 25 k. Still, some have MWs in a 60 k to 70 k range (Veraverbeke & Delcour, 2002). They contain higher levels of aspartic acid (asp)/asparagine (asn) (together referred to as asx), alanine (ala) and lysine (lys) and lower levels of glutamic acid (glu)/glutamine (gln) (together referred to as glx), and proline (pro) than gliadin and glutenin (Belitz, Grosch, & Schieberle, 2009).

Gliadin is subdivided in monomeric α -, γ - and ω -gliadin based on their amino acid (AA) sequences and MWs (Delcour, Joye, Pareyt, Wilderjans, Brijs, & Lagrain, 2012; Wieser, 2007).

64 The former (MWs about 28 k – 35 k) are more abundantly present and have lower MWs than
65 ω -gliadin (MWs about 34 k - 55 k) (Wieser, 2007). The N-terminal domains of α - and γ -gliadin
66 contain repetitive sequences rich in glx, pro and phenylalanine (phe) (Wieser, 2007), while
67 their non-repetitive C-terminal domains contain six and eight cysteine (cys) residues which
68 form three and four intramolecular disulfide (SS) bonds, respectively (Delcour, Joye, Pareyt,
69 Wilderjans, Brijs, & Lagrain, 2012; Wieser, 2007). ω -Gliadin lack cys. About 80% of the protein
70 consists of repetitive motives rich in glx, pro and phe (Belitz, Grosch, & Schieberle, 2009;
71 Shewry, Tatham, Forde, Kreis, & Mifflin, 1986; Wieser, 2007).

72 Glutenin is made up by SS interconnected glutenin subunits (GS) of high (HMW-GS, about 67 k
73 - 88 k) and low (LMW-GS, about 32 k – 39 k) MW (Belitz, Grosch, & Schieberle, 2009; Gianibelli,
74 Larroque, MacRitchie, & Wrigley, 2001; Veraverbeke & Delcour, 2002; Wieser, 2007). The
75 primary and secondary structures of LMW-GS are related to those of gliadin. However, they
76 have at least one additional cys residue which is involved in SS bond formation (Shewry &
77 Tatham, 1997; Wieser, 2007). The C- and N-terminal domains of HMW-GS contain one and
78 three to five cys residues, respectively (Gianibelli, Larroque, MacRitchie, & Wrigley, 2001;
79 Shewry, Halford, & Tatham, 1992). Their central repetitive domains are rich in glu, pro and
80 glycine (gly) (Belitz, Grosch, & Schieberle, 2009; Gianibelli, Larroque, MacRitchie, & Wrigley,
81 2001; Shewry, Halford, & Tatham, 1992).

82 Baking induces changes in wheat protein (Lagrain, Goderis, Brijs, & Delcour, 2010). At about
83 45 °C, hydrogen bonds break, conformational changes take place and hydrophobic patches
84 are exposed (Guerrieri, Alberti, Lavelli, & Cerletti, 1996). As a result of the increase in
85 temperature the strength of hydrophobic bonds increases which then stabilizes the protein
86 network (Wieser, 2007). Heating to 90 °C increases the MW of glutenin mainly through SS

oxidation and sulfhydryl (SH)-SS exchange reactions (Lagrain, Goderis, Brijs, & Delcour, 2010). At temperatures exceeding 90 °C α - and γ -gliadin become involved in the gluten network through SH-SS exchange reactions (Lagrain, Thewissen, Brijs, & Delcour, 2008) and wheat albumins and globulins also polymerize by SS bond formation. More wheat protein participates in heat-induced covalent network formation when gluten is combined with wheat albumin or globulin than when heating them separately. The level of accessible SH groups and the surface hydrophobicity of added globular proteins are key to enhancing heat-induced polymerization with gluten (Lambrecht, Rombouts, De Ketelaere, & Delcour, 2017). Little if any covalent cross-links other than SS bonds (*e.g.* those in lanthionine and lysinoalanine and isopeptide or dityrosine bonds) are formed during bread making (Lambrecht, Deleu, Rombouts, & Delcour, 2018).

Tanaka, Matsuzawa, and Ohta (1998) have postulated that Aq1 has three subsites in its substrate binding site with preferences for phe, valine (val), leucine (leu) and ala. However, whether Aq1 has preference for specific wheat proteins or domains in these proteins remains unknown.

We here for the first time investigated which proteins and, more specifically, which protein domains are affected by hydrolysis by Aq1 during bread baking. Changes in protein extractability in an SDS containing medium of heated dough from a control recipe or such recipe also containing Aq1 were studied. Next, proteins were extracted from bread crumb according to an Osborne type fractionation. Their MW distributions were studied under (non-)reducing conditions and the AA compositions of the extracted protein materials were determined. We here report on the outcome of this study.

2. Materials and methods

2.1. Materials

Crousti wheat flour [13.9% protein on dry matter (dm) basis] was from Dossche Mills (Deinze, Belgium) and Aq1 stock solution typically containing 6 units (U)/ μL (U defined in section 2.2) from Puratos (Groot-Bijgaarden, Belgium). Salt and sugar were from a local supermarket and fresh yeast from AB Mauri (Merelbeke, Belgium). All chemicals were of at least analytical grade. Sodium chloride, ethanol, SDS, dithiothreitol (DTT), urea, trichloroacetic acid, hydrochloric acid, disodium hydrogen phosphate, sodium dihydrogen phosphate and phenol were from VWR International (Leuven, Belgium). All other chemicals, molecular mass markers and AA standards were from Sigma Aldrich (Bornem, Belgium).

2.2. Aqualysin 1 activity assay

The activity of Aq1 was measured as in Verbauwhe et al. (2018). A mixture of Aq1 stock solution 150 times diluted in water (25 μL), deionized water (25 μL), sodium carbonate buffer (70 μL ; 50 mM; pH 10.0) and substrate solution (70 μL ; 1.6 mg N-succinyl-ala-ala-pro-phe-*p*-nitroanilide in 1.0 mL of the same buffer) was incubated at 50 °C for 20 min. The absorbance of 100 μL sample was then colorimetrically measured at 415 nm (room temperature; Microplate Reader Model 680, Bio-Rad, Hercules, CA, USA). Absorbance values were corrected for that of substrate in absence of Aq1. A 1.0 U of Aq1 activity yields a corrected absorbance of 1.000 under these assay conditions.

2.3. Bread making

Straight-dough breads were made essentially as in Finney (1984). Wheat flour (100.0 g; 14.0% moisture), yeast (5.30 g), sodium chloride (1.50 g), sugar (6.00 g), calcium propionate (0.25 g) and deionized water [59.0 mL; *i.e.* Farinograph water absorption determined with AACC

method 38-20.01 (AACC, 1999)] without (control) or with Aq1 (150 ± 5 U/g flour protein) were mixed for 225 s [optimal mixing time obtained from Mixograph analysis with AACC method 54-40.02 (AACC, 1999)] at 23 °C in a 100 g pin mixer (National Manufacturing, Lincoln, NE, USA). Dough was fermented (90 min; 30 °C; 90% relative humidity) in a fermentation cabinet (National Manufacturing) and sheeted after 52 min and 77 min. It was then sheeted, molded, proofed (36 min; 30 °C; 90% relative humidity) and baked (24 min; 215 °C) in a rotary oven (National Manufacturing). Bread was allowed to cool to room temperature prior to sampling. In addition, aliquots of proofed dough (about 0.6 g) were heated in closed containers at temperatures ranging from 50 °C to 95 °C for 15 min and cooled on ice. Heated dough and bread samples were freeze dried, ground and sieved (250 μ m). Samples are further referred to as freeze dried dough or crumb.

2.4. Fractionation of bread crumb proteins

Proteins were extracted from freeze dried crumb (4.00 g) by shaking (10 min; 150 rpm; 4 °C) in sodium phosphate buffer (20.0 mL; 0.05 M; pH 6.8) containing both NaCl (0.4 M) and the serine peptidase inhibitor phenylmethylsulfonyl fluoride (PMSF; 40 mg/L) and subsequent centrifugation (10 min; 10,000 g ; 4 °C). Two additional extractions from the pellet were performed. Prior to doing so, the pellets were disintegrated into small pieces. All collected supernatants were freeze dried, ground and sieved (250 μ m). The pellets were shaken (10 min; 150 rpm; 4 °C) with 60% (v/v) ethanol (12.0 mL) containing PMSF (40 mg/L). After centrifugation as above, pellets were disintegrated into small pieces. After two additional extractions of the disintegrated pellets, solvents were evaporated under reduced pressure at 40 °C (Rotavapor, R-300, Buchi, Flawil, Switzerland) and the residues were ground and sieved (250 μ m).

2.5. Isolation of gluten, gliadin and glutenin from wheat flour

Gluten was isolated from wheat flour essentially as in Frederix, Courtin and Delcour (2004). Wheat flour (250 g) and 140 mL deionized water were mixed (4 min) with a KitchenAid (St. Joseph, MI, USA) mixer KPM5 equipped with a dough hook. After an 8 min rest, 250 mL deionized water was added and the dough was transformed into batter by stirring (25 min) with a flat beater. Deionized water (1 L) was then added and the suspension stirred (35 min) and then sieved (125 μ m; 5 min; 70 Hz) while being vibrated. The gluten retained on the sieve was washed two times with deionized water (1 L), collected, freeze dried, ground and sieved (250 μ m).

Gliadin and glutenin were isolated from gluten with an Osborne type fractionation procedure (Osborne, 1907). Gluten samples containing 20.0 g protein (see 2.7) were shaken (150 rpm; 60 min) with 250 mL 0.05 M sodium phosphate buffer (pH 6.8) containing 0.4 M NaCl and centrifuged (10 min; 10,000 g). The pellets were disintegrated into small pieces and most of the salt removed with deionized water (250 mL) by shaking (150 rpm; 60 min) and centrifugation (10 min; 10,000 g). Proteins were then sequentially extracted from the pellets with 70% (v/v) ethanol (250 mL) and 60% (v/v) ethanol (250 mL). Each time, the pellets were first divided into small pieces, shaken (150 rpm; 60 min) with the ethanol solution and centrifuged (10 min; 10,000 g). The 60% (v/v) ethanol extracts were discarded. The 70% (v/v) ethanol extracts were filtered over filter paper. Ethanol was evaporated under reduced pressure at 40 °C as above. Samples were freeze dried, ground and sieved (250 μ m). The materials obtained from the 70% ethanol extracts are further referred to as gliadin, those from the pellets after the sequential extraction steps as glutenin.

2.6. Isolation of low and high molecular weight glutenin subunits

LMW-GS and HMW-GS were purified from glutenin essentially as in Verbruggen, Veraverbeke, Vandamme, and Delcour (1998). Glutenin (2.0 g) suspended in 400 mL 50% (v/v) 1-propanol containing DTT [0.1% (w/v)] was stirred (30 min; 60 °C) and centrifuged (20 min; 4,600 g). HMW-GS were precipitated from the supernatant by bringing the 1-propanol concentration to 60% (v/v) and centrifugation (20 min; 4,600 g) after a 30 min rest (room temperature). The supernatant and pellet contained LMW-GS and HMW-GS respectively. 1-Propanol was evaporated as above. All samples were then freeze dried, ground and sieved (250 µm).

2.7. Protein and moisture contents

The protein content (N x 5.7) of wheat flour, fractions of freeze-dried crumb obtained by sequential extraction with salt and ethanol solutions (section 2.5.) as well as gliadin and glutenin (section 2.6) were determined with AOAC method 990.03 (AOAC, 1995) adapted to an automated Dumas protein analysis system (EAS Vario Max Cube, Elementar, Hanau, Germany). Moisture contents were determined with AACC method 44-15.02 (AACC, 1999).

2.8. Determination of the amino acid composition

The AA compositions of the proteins in the freeze-dried extracts of Sections 2.5. and 2.6 were determined by acid hydrolysis and subsequent quantification with high performance anion-exchange chromatography with pulsed amperometric detection essentially as in Rombouts, Lamberts, Celus, Lagrain, Brijs and Delcour (2009). (Iso-)peptide bonds in samples containing 10.0 mg protein were hydrolyzed under nitrogen atmosphere by heating at 110 °C for 24 h in 4.0 mL 6.0 M HCl containing 1.0 g/l phenol and norleucine (1.5 mM; internal standard). Diluted hydrolysates (400 fold) were filtered (Millex-GP, 0.22 µm, polyethersulfone, Millipore, Carrigtwohill, Ireland) and analyzed with a Dionex BioLC system (Sunnyvale, CA, USA).

Calculations and correction factors for val (1.07) and serine (ser, 1.32) were as in Rombouts, Lamberts, Celus, Lagrain, Brijs and Delcour (2009).

2.9. Size exclusion high performance liquid chromatography

The MW distribution of glutenin and gliadin (section 2.5.) and proteins extracted from freeze dried crumb produced from control and Aq1 containing recipes (section 2.4.) were determined in triplicate with size exclusion high performance liquid chromatography (SE-HPLC). Samples containing 1.0 mg protein were extracted (60 min; 150 rpm) with 1.0 mL 50 mM sodium phosphate buffer (pH 6.8) containing 2.0% (w/v) SDS and 40 mg/L PMSF and centrifuged (10 min; 10,000 *g*). The latter extraction medium not containing PMSF was used as eluent. Extraction under reducing conditions was performed under nitrogen atmosphere with the same sodium phosphate buffer containing 2.0% (w/v) SDS, 40 mg/L PMSF, 2.0 M urea and 1.0% (w/v) DTT. The extracted proteins (20 μ L) were separated with a Biosep-SEC-S3000 (pore size 29 nm, Phenomenex, Torrance, CA, USA) column with exclusion range of 5 k to 100 k. Proteins were detected at 214 nm with a Shimadzu (Kyoto, Japan) LC-2010 system. The level of protein extractable in SDS containing medium under non-reducing conditions (SDS-EP-NR) from control or Aq1 dough was calculated from the corresponding peak areas and expressed as a percentage of total peak area, *i.e.* the area of respectively control or Aq1 dough sample recorded under reducing conditions. Protein markers were chicken egg white ovalbumin (45 kDa), anhydrase (30 kDa), ribonuclease A (14 kDa), insulin B (4 kDa).

2.10. Statistical analysis

All analytical analysis (determination of AA and protein contents, SE-HPLC) were at least performed in triplicate. Significance of differences ($\alpha = 0.05$) based on at least three

measurements was determined with a student T test using JMP Pro 11.2.0 (SAS Institute, Cary, NC, USA).

3. Results and discussion

3.1. The impact of aqualysin 1 use on the level of protein extractable in sodium dodecyl sulfate containing medium in heated dough

Under non-reducing conditions, about 90% of all proteins in unheated control dough were extractable (**Fig. 1.**). The SE-HPLC profile of the latter is subdivided in populations (P) I to IV, eluting before 14 min 30 sec (P. I), between 14 min and 16 min 15 sec (P. II), between 16 min 15 sec and 18 min 30 sec (P. III) and after 18 min 30 sec (P. IV) (**Fig. 2.A**). P. I and P. II mainly contain glutenin and ω -gliadin, respectively, P. III mainly α - and γ -gliadin proteins and P. IV mainly albumin and globulin. Heating aliquots of fermented dough to 65 °C for 15 min impacted neither the SDS-EP-NR level (**Fig. 1.**) nor the MW distribution (profiles not shown) of control dough. In this temperature range, mainly non-covalent interactions are affected (Guerrieri, Alberti, Lavelli, & Cerletti, 1996; Lefebvre, Popineau, Deshayes, & Lavenant, 2000; Weegels, de Groot, Verhoek, & Hamer, 1994) which have no impact on the extractability of the proteins in SDS containing medium. At temperatures exceeding 70 °C the level of SDS-EP-NR significantly decreased as a result of SS bond formation (**Fig. 1.**). In contrast to what was the case for the levels of the other fractions, that of P. I significantly decreased as a result of heating at 70 °C and 75 °C due to glutenin polymerization and concomitant loss of its extractability (**Fig. 2.A**). At temperatures exceeding 90 °C, the extractability of α - and γ -gliadin under non-reducing conditions decreased, as noted in P. III. These higher temperatures lead to incorporation of gliadin in the gluten network mainly through SH-SS exchange reactions (Lagrain, Thewissen, Brijs, & Delcour, 2008). The slight heat-induced reduction of the level of

P. II material is due to polymerization of some glutenin and α - and γ -gliadin proteins co-eluting with ω -gliadin (**Fig. 2.A.**). The relative contents of albumin and globulin proteins (P. IV) were also negatively affected by heating. They contain higher levels of free SH groups than gluten proteins. This and the presence of hydrophobic protein patches enhance protein network formation which involves both gluten and globular proteins (Lambrecht, Rombouts, De Ketelaere, & Delcour, 2017).

The SDS-EP-NR levels in samples that contained Aq1 decreased when exposed to temperatures exceeding 70 °C (**Fig. 1**). This was mainly the result of SS bond formation. Aq1 use resulted in higher levels of SDS-EP-NR than for control samples when both heated to at least 80 °C. Indeed, for samples heated to 95 °C in which Aq1 had been used, the SDS-EP-NR level was approximately 15% higher than for comparable samples in which no Aq1 had been used. In bread making, Aq1 use has been reported to result in 20% higher SDS-EP-NR contents (Verbauwhede, et al., 2018). Although the total SDS-EP-NR level in samples heated to 85°C was higher when Aq1 had been applied (**Fig. 1.**), lower levels of glutenin and gliadin were extracted due to protein hydrolysis and the accompanying shift from high to low MW (**Fig. 2.B.**). Indeed, the lower levels of SDS-EP-NR in P. I and P. II were compensated by higher levels in P. IV (**Fig. 2.B.**) at 85 °C. For samples treated at temperatures exceeding 85 °C, the same trends were noted. Thus, the total level of SDS-EP-NR was mainly higher due to peptides occurring in P. IV.

3.2. Osborne type fractionation of crumb from control or aqualysin 1 containing recipe

In general, gluten protein hydrolysis increases their solubility and extractability in water and salt solutions (Wouters, Rombouts, Fierens, Brijs, & Delcour, 2016). Wheat flour gliadin and GS are extractable with ethanol solution (Osborne, 1907; Veraverbeke & Delcour, 2002).

3.2.1. Protein extracted with salt solution

Salt solution extracted only about 4% of all protein from control bread crumb. Most extracted proteins eluted in P. IV (**Fig. 3.A.1.**). The dilute salt extract was mainly rich in glx, pro, ala, ser and leu containing protein sequences (**Table 1.**). The AA composition and the MW distribution confirm the extraction of mainly albumin and globulin with dilute salt from control crumb (Belitz, Grosch, & Schieberle, 2009).

In contrast, salt solution extracted about 31% of proteins and peptides from crumb when the bread recipe contained Aq1. Evidently, not all of this protein material was albumin or globulin, but also some hydrolyzed gluten. Most of the extracted proteins and peptides eluted in P. IV (**Fig. 3.B.1.**). A minor fraction had MWs around 30 k but most proteins and peptides now had MWs between 14 k and 4 k. Inclusion of DTT and urea in the SDS solution used for solubilizing the freeze dried extract had a negligible impact on the MW distribution. This showed that little if any of the peptides were interconnected by SS bonds.

At the same time, the salt solution extractable proteins were enriched in glx and phe and had less asx, lys, ala, threo and val when Aq1 had been applied in the bread making process (**Table 1.**). Whereas glx and phe are abundantly present in the repetitive domains of gluten proteins, asx, lys, ala, threo and val are not (Belitz, Grosch, & Schieberle, 2009). The levels of pro, ser and leu were similar irrespective of whether Aq1 had been used or not. These AAs occur in albumin, globulin and the repetitive domains of gluten proteins (Belitz, Grosch, & Schieberle,

2009). Overall, as a result of Aq1 use, the dilute salt extract was enriched in peptides that mainly originate from the repetitive domains of gluten proteins.

3.2.2. Protein extracted with ethanol solution

The subsequently used aqueous ethanol solution extracted about 6% of all protein from control crumb. These proteins eluted in P. II and P. III and are mainly ω -gliadin and α - and γ -gliadin, respectively (**Fig. 3.A.2.**). Under reducing conditions, their MW distribution was comparable to that of isolated gliadin (**Figs. 3.A.2. and 4.A.**). The shift in elution time from 17 min 15 sec to 16 min 45 sec is due to the higher hydrodynamic volume of α - and γ -gliadin as a result of the reduction of their intramolecular SS bonds (Beckwith, Wall, & Jordan, 1965; Woychik, Dimler, & Huebner, 1964) (**Fig. 3.A.2.**). ω -Gliadin was more abundant in the aqueous ethanol extract than were α - and γ -gliadin as it lacks the ability to polymerize through SS bond formation.

When Aq1 was applied, about 4% of all proteins were extracted with aqueous ethanol. Reduction had no clear impact on the MW distribution (**Fig. 3.B.2.**). The proteins of P. II and the left peak of P. III were based on their MW (**Figs 3.B.2. and 4.A.**) and extractability in aqueous ethanol solution probably intact ω -, α - and γ -gliadin. As only negligible amounts of gliadin and GS (**Fig. 4.A and 4.B.**) eluted in P. IV, the right peak of P. III and P. IV (**Fig. 3.**) most likely represents peptides from gluten proteins which were not incorporated in the heat-induced covalent protein network.

3.2.3. Protein not extracted with salt and ethanol solutions

Approximately 90% of all proteins in control crumb were not extractable with salt and ethanol solutions. Also, because of extensive polymerization at higher temperatures, almost none of

this protein was extractable with SDS containing medium under non-reducing conditions (Fig. 3.A.3.). Comparison of the MW distribution of these proteins under reducing conditions with those of glutenin and gliadin (Figs. 3.A.3., 4.A. and 4.B.) indicates that this protein mainly was gluten. The main constituting AAs were glx, pro, phe and leu with lower levels of lys, ala and asx than noted for the extract prepared with the salt solution (Table 1.).

Even when Aq1 had been applied SDS containing medium without reducing agent extracted hardly any protein from the pellet resulting after sequential extraction with salt and ethanol solutions (Fig. 3.B.3.). That reducing conditions drastically increased protein extractability both from control samples and samples produced with Aq1 use indicates that the protein network in the crumb of bread from an Aq1 containing recipe also contained SS interconnected proteins and peptides. The proteins in P. II and the peak at about 17 min of P. III had MW distributions comparable to those of the proteins extracted neither with salt nor with aqueous ethanol solutions from control bread crumb (Fig. 3.3.A.) and the isolated gliadin (Fig. 4.A.) and LMW-GS (Fig. 4.B.). Aq1 acted on almost all HMW-GS in the glutenin structure as evidenced from comparison of Figs. 3.B.3 with Fig. 3.A.3. and 4.B. and the resultant hydrolysis products will evidently elute at lower MW. Peptides released from the thermoset gluten network under reducing conditions from crumb of bread prepared with use of Aq1 had MWs around 30 k and lower than 14 k (Fig. 3.B.3.). The composition of the aqueous ethanol extract of crumb from bread made with the Aq1 containing recipe (Fig. 3.B.2.) indicated that only a minor fraction of peptides and non-hydrolyzed gliadin and LMW-GS were not covalently incorporated in the heat-induced protein network. Aq1 use caused the heat-induced covalent network to have significantly lower levels of glx, pro and phe, and at the same time higher levels of lys, ala, val and asx. Lys, ala, val and asx rarely occur in the repetitive domains of gluten proteins but are present in their non-repetitive domains (Table 1.). Overall, Aq1 use

results in a thermoset gluten network that mainly consists of polymerized non-repetitive domains of gluten proteins and minor levels of non-hydrolyzed gliadin and LMW-GS (**Fig. 5.**).

4. Conclusion

The impact of gluten protein hydrolysis by Aq1 during bread baking was investigated by Osborne type sequential extraction of proteins and peptides from crumb. Aq1 use in bread making caused the salt solution to extract significantly more protein from crumb. This extract was enriched in peptides from the repetitive domains of the gluten proteins. These peptides also accounted for the higher SDS-EP-NR content as a result of Aq1 use when samples had been treated to temperatures exceeding 80 °C. Furthermore, Aq1 use resulted in the crumb polymeric protein to mainly consist of peptides originating from the non-repetitive domains of the gluten proteins. Some intact gliadin and LMW-GS presumably also occur in the thermoset gluten network. The enzymatic formation of cys containing peptides occurs in the same temperature range as that in which protein polymerization occurs. The mobility and molecular behavior of these cys containing peptides is unknown. Therefore, the impact of Aq1 use on the heat-induced protein microstructure remains to be investigated.

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443

444 **Figures**

445
446 **Figure 1:** Aliquots of control (black bars) and aqualysin 1 (Aq1, white bars) containing dough
447 were heated for 15 min at various temperatures. The level of protein extractable in sodium
448 dodecyl sulfate containing medium under non-reducing conditions (SDS-EP-NR) from control
449 or Aq1 dough was calculated from the corresponding peak areas and expressed as a
450 percentage of total peak area, *i.e.* the area of respectively control or Aq1 dough sample
451 recorded under reducing conditions. Standard deviations are indicated with error bars.

Figure 2: Aliquots of control (A.) and aqualysin 1 (Aq1) containing (B.) dough were heated for 15 min at various temperatures. Proteins were extracted with sodium dodecyl sulfate (SDS) containing medium under non-reducing conditions (SDS-EP-NR) and separated with size exclusion high performance liquid chromatography (SE-HPLC). The profiles are divided in populations P. I, P. II, P. III and P. IV. The molecular weights (MWs) of markers (45 k, 30 k, 14 k and 4 k) are indicated at the top of the figure. Some proteins and peptides eluted past the exclusion range of the column (5 k – 100 k) given by the supplier. AU, arbitrary units.

Figure 3: Proteins were sequentially extracted with salt (1.) and ethanol (2.) solutions from bread produced from control (A.) and aqualysin 1 (Aq1) containing (B.) dough. Protein not extractable with salt and ethanol solutions was retained in the pellet (3.). Freeze-dried samples were solubilized with sodium dodecyl sulfate (SDS) containing medium under reducing (full line) and non-reducing (dotted line) conditions and separated with size exclusion high performance liquid chromatography (SE-HPLC). The profiles are divided in populations I, II, III and IV. The molecular weights (MWs) of markers (45 k, 30 k, 14 k and 4 k) are indicated at the top of the figure. Some proteins and peptides eluted past the exclusion range of the column (5 k – 100 k) given by the supplier. AU, arbitrary units.

Figure 4: Gliadin (A., dotted black line) and glutenin (A., full grey line) were isolated from wheat flour. High molecular weight (HMW, B., full light grey line) and low molecular weight (LMW, B. full dark grey line) glutenin subunits (GS) were then obtained from glutenin. The proteins and GS were separated with size exclusion high performance liquid chromatography (SE-HPLC) after solubilizing in a sodium dodecyl sulfate (SDS), dithiothreitol (DTT) and urea containing medium. The SE-HPLC profiles are divided in populations I, II, III and IV. The molecular weights of some markers (45 k, 30 k, 14 k and 4 k) are indicated on top of the profiles with numbers. Some proteins and peptides eluted past the exclusion range of the column (5 k – 100 k) given by the supplier. AU, arbitrary units.

Figure 5: Schematic representation of high molecular weight (HMW) and low molecular weight (LMW) glutenin subunits (GS) and α-, γ-gliadin and ω-gliadin. Repetitive and non-repetitive domains are respectively colored in grey and white (Delcour, Joye, Pareyt, Wilderjans, Brijs, & Lagrain, 2012). The frequently occurring repetitive amino acid (AA) sequences are indicated with their conventional one-letter codes in black (Belitz, Grosch, & Schieberle, 2009; Wieser, 2007). Cysteine residues are indicated in grey with “c” and the additional ones in γ-type HMW-GS compared to x-type HMW-GS and the additional ones in γ-gliadin compared to α-gliadin are indicated by “c*” (Delcour, Joye, Pareyt, Wilderjans, Brijs, & Lagrain, 2012). Hydrolysis of gluten proteins by aqualysin 1 (Aq1) is presented in a simplified way and is only given to illustrate the presence of peptides from repetitive domains in the salt solution extract and of non-repetitive domains in the covalent heat-induced protein network. The resulting peptides are indicated by dotted lines. Molecular weights (MWs) of the gluten proteins (Wieser, 2007) are indicated between brackets.

498 Table

499 **Table 1:** Proteins were sequentially extracted with salt and aqueous ethanol solutions from
500 bread produced from control and aqualysin 1 (Aq1) containing dough. Protein not extractable
501 with salt and aqueous ethanol solutions were retained in the pellet. Concentrations of some
502 amino acids (AAs) in the salt extract and pellet are given in $\mu\text{mol/g}$ protein. Standard
503 deviations are given between brackets. Significant differences ($\alpha = 0.05$) between bread
504 crumb without or with inclusion of Aq1 for the same fraction are indicated by *.

	Salt solution		Pellet	
	Control	Aq1	Control	Aq1
Lysine	342 (39)*	114 (5)*	178 (7)*	216 (5)*
Alanine	670 (79)*	303 (4)*	190 (12)*	443 (13)*
Threonine	315 (19)*	212 (4)*	279 (7)*	306 (6)*
Valine	336 (17)*	281 (6)*	433 (16)*	485 (8)*
Serine	606 (41)	581 (12)	589 (15)	588 (11)
Proline	1363 (109)	1425 (25)	1108 (27)*	976 (28)*
Leucine	447 (20)	440 (23)	688 (16)	765 (19)*
Phenylalanine	259 (35)*	425 (7)*	417 (6)	412 (5)
Glutamic acid / Glutamine	1515 (94)*	2054 (37)*	1647 (37)*	1495 (38)*
Aspartic acid / Asparagine	319 (26)*	141 (1)*	235 (7)*	282 (12)*

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