Thermo-reversible inhibition makes aqualysin 1 from *Thermus aquaticus* a potent tool for studying the contribution of the wheat gluten network to the crumb texture of fresh bread

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Running title: Impact of Aqualysin 1 on the gluten network and texture of fresh bread

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Keywords: peptidase; serine peptidase inhibitors; thermo-active; bread; gluten functionality

23

24 **Abstract**

25 The thermo-active serine peptidase aqualysin 1 (Aq1) of *Thermus aquaticus* was applied in bread
26 making to study the relative contribution of thermoset gluten to bread crumb texture. Aq1 is
27 active between 30 °C and 90 °C with an optimum activity temperature of around 65 °C. It is
28 inhibited by wheat endogenous serine peptidase inhibitors during dough mixing and
29 fermentation and starts hydrolyzing gluten proteins during baking above 80 °C when the enzyme
30 is no longer inhibited and most of the starch is gelatinized and contributes to structure formation.
31 Aq1 activity reduced the molecular weight of gluten proteins and significantly increased their
32 extractability in sodium dodecyl sulfate containing medium. While it had no impact on the
33 specific bread volume and only limited impact on hardness, cohesiveness, springiness, resilience
34 and chewiness, it impacted bread crumb coherence. We conclude that starch has a greater
35 impact on crumb texture than thermoset gluten.

36 **1. Introduction**

37 The unique bread making properties of wheat are ascribed to the visco-elastic properties of its
38 gluten proteins. They make up about 80% of the total protein content and are divided in two
39 groups: monomeric gliadins [molecular weight (MW) of 30 - 80 k] and polymeric glutenins (MW
40 of 80 k to more than twenty million) (Shewry, Tatham, Forde, Kreis, & Mifflin, 1986; Veraverbeke
41 & Delcour, 2002). Gliadins are subdivided in α -, γ - and ω -gliadins. The α - and γ -gliadins contain
42 cysteine residues which are connected through intramolecular disulfide (SS) bonds. ω -Gliadins
43 lack cysteine residues (Delcour, Joye, Pareyt, Wilderjans, Brijs, & Lagrain, 2012; Shewry, Tatham,

44 Forde, Kreis, & Mifflin, 1986; Wieser, 2007). Glutenin consists of subunits (GS) which are
45 interconnected by SS bonds. GS contain intramolecular SS bonds and have at least one (free)
46 cysteine residue which enables them to form intermolecular SS bonds (D'Ovidio & Masci, 2004;
47 Lindsay & Skeritt, 1999; Megan & Skeritt, 1999; Shewry & Tatham, 1997; Shewry, Tatham,
48 Forde, Kreis, & Mifflin, 1986).

49 When wheat flour is mixed with an appropriate amount of water, a cohesive visco-elastic gluten
50 network is formed (H. Singh & MacRitchie, 2001; Song & Zheng, 2007; Veraverbeke & Delcour,
51 2002). This network retains the gas produced by yeast during fermentation and expansion.

52 During baking, at temperatures below 40 °C reversible changes take place (Lefebvre, Popineau,
53 Deshayes, & Lavenant, 2000). At higher temperatures (up to 90 °C), glutenin forms a network
54 based on intermolecular SS bonds (Delcour & Hosney, 2010; Lagrain, Brijs, Veraverbeke, &
55 Delcour, 2005; Schofield, Bottomley, Timms, & Booth, 1983). At temperatures exceeding 90 °C,
56 in addition to the above, gliadins are covalently incorporated into the gluten structure mainly
57 through sulfhydryl (SH)-SS exchange reactions (Lagrain, Thewissen, Brijs, & Delcour, 2008).

58 In the baking process, starch gelatinizes and a thermoset gluten network is formed. Starch
59 gelatinization [i.e. loss of its birefringent properties (Delcour & Hosney, 2010)] and the
60 phenomena which accompany it (including swelling of the starch granules, water uptake, and
61 leaching of amylose) result in a sharp increase in viscosity and migration of plasticizing water
62 from the gluten network to starch (Delcour & Hosney, 2010; Goesart, Brijs, Veraverbeke,
63 Courtin, Gebruers, & Delcour, 2005; A. P. Singh & Bhattacharya, 2005). The further expansion of
64 carbon dioxide gas and evaporation of ethanol and water cause rupture of the starch-protein

65 matrix that surrounds the gas cells. These phenomena cause the transformation of a dough (a
66 foam) into a gas continuous crumb by the end of the oven rise (Delcour & Hoskeney, 2010; Fan,
67 Mitchell, & Blanshard, 1999; Goesaert, Brijs, Veraverbeke, Courtin, Gebruers, & Delcour, 2005;
68 Scanlon & Zghal, 2001; A. P. Singh & Bhattacharya, 2005). Although the changes in starch and
69 gluten proteins have been described, little is known about the relative contribution of both
70 polymer types to bread crumb texture.

71 Endopeptidases generally already hydrolyze gluten to some degree during dough mixing and
72 fermentation. Their action impairs dough rheology, its gas retention capacity and the crumb
73 structure and specific volume of the resultant bread. To avoid dough structure collapse before
74 oven spring, peptidase activity should be limited. However, appropriate peptidase use can result
75 in soft fresh bread crumb and delay crumb firming and the subsequent increase of its chewiness
76 during storage (Caballero, Gomez, & Rosell, 2007; Indrani, Prabhasankar, & Venkateswara Rao,
77 2003; Mathewson, 2000). In the present work, we used the thermo-active endopeptidase
78 aqualysin 1 (Aq1; EC 3.4.21.111) for studying the importance of the gluten network for bread
79 texture. This alkaline serine peptidase which belongs to the subtilisin-type peptidases is secreted
80 by the thermophilic bacteria *Thermus aquaticus*. At pH 6 and 7, Aq1 has respectively about 40%
81 and 60% of its optimal activity at pH 10 (Matsuzawa, Tokugawa, Hamaoki, Mizoguchi, Taguchi,
82 Terada, et al., 1988). Aq1 is optimally active at 70 °C (Matsuzawa, et al., 1988; Rawlings, Barrett,
83 & Bateman, 2010) which would imply that it is active in the time frame during which starch
84 gelatinization, dough / crumb transition and gluten polymerization occur. Also, as wheat flour
85 contains serine peptidase inhibitors (Di Maro, Farisei, Panichi, Severino, Bruni, Ficca, et al., 2011;
86 Mundy, Hejgaard, & Svendsen, 1984; Odani, Koide, & Ono, 1986; Østergaard, Rasmussen,

Roberts, & Hejgaard, 2000; Poerio, Di Gennaro, Di Maro, Farisei, Ferranti, & Parente, 2003), we speculated that its activity during the dough and early baking stages would be very limited and that its inhibition, if any, would be thermo-reversible. In other words, we reasoned that, if the above proved to be true, use of Aql may help us to understand the importance of the thermoset gluten network for bread crumb and texture. To that end, we investigated the Aql activity at different temperatures in the presence of wheat flour proteins extracted in a dilute salt solution and its impact on gluten network formation during baking and on bread crumb texture.

2. Materials and methods

2.1. Materials

Crousti wheat flour [12.0% protein; 14.0% moisture] was from Dossche Mills (Deinze, Belgium) and commercial gluten [Amygluten; 77.0% protein (N x 5.7); 6.0% moisture] from Syral (Aalst, Belgium). Salt and sugar were from a local supermarket. Fresh yeast was from AB Mauri (Merelbeke, Belgium). All chemicals were at least of analytical grade. Acetic acid, sodium dodecyl sulfate (SDS), dithiothreitol (DTT), urea, disodium hydrogen phosphate and sodium dihydrogen phosphate were from VWR International (Leuven, Belgium). Bovine milk α -lactalbumin was from Acros Organics (Geel, Belgium). All other chemicals, reagents and molecular mass markers were from Sigma-Aldrich (Bornem, Belgium). Aql was from Puratos (Groot-Bijgaarden, Belgium).

2.2. Determination of moisture and protein content

Moisture content was determined according to AACC method 44-15.02 (AACC). Protein contents were obtained by an adaptation of the AOAC method 990.03 (AOAC, 1995) to an automated Dumas protein analysis system (EAS Vario Max Cube, Elementar, Hanau, Germany).

2.3. The impact of wheat flour extract on aqualysin I activity

The presence or absence of serine peptidase inhibitors in wheat flour was investigated with an assay. Wheat flour extract (WFE) was prepared by shaking (15 min) 15.0 g wheat flour with 100 ml sodium acetate buffer (50 mmol/l; pH 5.5) and recovered by centrifugation [15 min; room temperature (RT); 3,000 g]. It contained 1.8 mg/ml ($\pm$ 0.1 mg/ml) protein (N x 6.25). Aq1 solution (25 μ l containing 1.00 U of enzyme activity, see below for its definition) was preincubated with 25 μ l deionized water or WFE for 30 min. Next, 70 μ l sodium carbonate buffer (50 mmol/l; pH 10.0) and 70 μ l substrate solution (1.6 mg N-succinyl-ala-ala-pro-phe-*p*-nitroanilide in 1.0 ml of the same buffer) were added. After incubation for 20 min at different temperatures, the absorbance of *p*-nitroaniline was measured colorimetrically at 415 nm (100 μ l in a microtiter plate at RT). Under these assay conditions, 1.0 U Aq1 is the amount of enzyme which at 50 °C and in the presence of water instead of WFE results in an absorbance value of 1.000. Absorbance values were converted to concentrations of *p*-nitroaniline using a calibration curve constructed with the same buffer. The absorbance of each sample was corrected for the absorbance of the substrate in absence of Aq1 (50 μ l deionized water, 70 μ l buffer and 70 μ l substrate solution).

2.4. Bread making

Breads were made according to a straight-dough method (Finney, 1984). The dough recipe consisted of wheat flour (100.0 g; 14.0% moisture), 5.30 g yeast, 1.50 g sodium chloride, 6.00 g sugar, 59.0 ml deionized water [i.e. the Farinograph water absorption determined with AACC method 38-20.01 (AACC)] and 0.25 g calcium propionate. Aq1 dough contained 150 ± 5 U/g flour protein. Mixing for 225 s [optimal mixing time obtained from Mixograph analysis with AACC

method 54-40.02 (AACC)] at 23 °C in a 100 g pin mixer (National Manufacturing, Lincoln, NE, USA) was followed by fermentation (90 min; 30 °C; 90% relative humidity) in a fermentation cabinet (National Manufacturing). After 52 min and 77 min of fermentation, the dough was passed through a dough sheeter (National Manufacturing). Fermentation was followed by final sheeting, molding and proofing (36 min; 30 °C; 90% relative humidity). After proofing, doughs were baked (24 min; 215 °C) in a rotary oven (National Manufacturing) and cooled to RT over a 150 min period. Bread volume was measured with a VolScan Profiler (Stable Micro Systems, Goldaming, UK). In the model system designed to determine the onset temperature of Aq1 activity, aliquots of proofed dough (0.6 g) were kept in closed containers at temperatures ranging from 50 °C to 95 °C for 15 min and immediately cooled on ice.

2.5. Rapid visco analyzer analysis

The impact of Aq1 on the properties of gluten during heat-treatment was investigated with a rapid visco analyzer (RVA) essentially as in Lagrain et al. (2005). To a quantity of commercial gluten corresponding to 4.25 g protein (N x 5.7) in a RVA cup, water was added to obtain a total weight of 25.00 g. When Aql solution (11.0 or 22.0 U/g protein) was added, this was done prior to adding water to obtain a total weight as above. After homogenizing the suspension in an RVA-4D (Newport Scientific, Sydney, Australia) (20 s; 900 rpm) device, it was subjected to viscosity measurement with a temperature-time profile that mimics bread baking. In short, samples were first heated to 40 °C in 1 min and then to 95 °C in 14 min. They were subsequently held at 95 °C for 40 min and cooled to 50 °C in 7 min. The temperature profile ended with a holding phase at 50 °C for 13 min. During this temperature-time profile, the RVA paddle was rotated at 160 rpm.

2.6. Baking of model systems

To investigate the role of both starch and gluten during bread crumb formation, dough balls containing only gluten (GD) or gluten and starch (GSD) were made. Gluten dough (GD) samples made from commercial gluten (8.6 g dm) and 4.2 ml water or Aql solution containing 150 ± 5 U/g gluten protein were kneaded by hand (210 s; 23 °C) and baked (13 min; 232 °C) in a rotary oven (National Manufacturing). Gluten-starch doughs (GSD) consisted of commercial gluten (2.15 g dm), starch (6.45 g dm) and 6.32 ml water or Aql solution containing 150 ± 5 U/g gluten protein. They were mixed (330 s) in a 10 g pin mixer (National Manufacturing) and baked (13 min; 232 °C) in a rotary oven (Model Hearth, National Manufacturing). Pictures of the baked products were taken the next day.

2.7. Size exclusion high performance liquid chromatography

Dough, fermented dough and bread crumb were frozen in liquid nitrogen, freeze dried, grinded and sieved (250 μ m). Samples (1.0 mg protein) were extracted with 1.0 ml solution A [sodium phosphate buffer (50 mmol/l; pH 6.8) containing 2.0% SDS and 40.0 mg/l phenylmethylsulfonyl fluoride] or solution B [i.e. solution A also containing 2.0 M urea and 1.0% DTT]. Extraction with solution B was performed under nitrogen atmosphere to avoid DTT oxidation. Samples were shaken (60 min; RT) and centrifuged (10 min; RT; 10,000 g). The resultant supernatants were filtered (Millex-HP, 0.45 μ m, polyether-sulfone, Millipore, Carrigtwohill, Ireland) and aliquots (20 μ l) separated by size exclusion high performance liquid chromatography (SE-HPLC) (LC-2010 system; Shimadzu, Kyoto, Japan) on a Biosep-SEC-S4000 (pore size 500 Å, Phenomenex, Torrance, CA, USA) column. The eluent was sodium phosphate buffer (50 mmol/l; pH 6.8) containing 2.0%

SDS (flow rate 1.0 ml/min; 30 °C). Elution was monitored at 214 nm. Protein extractable in SDS containing medium (SDS-EP) was expressed as a percentage of total peak area of proteins extracted from control dough under reducing conditions. Protein markers were ribonuclease A (13.7 k), bovine milk α -lactalbumin (14.4 k), soybean trypsin inhibitor (20.2 k), α -chymotrypsinogen A from bovine pancreas (25 k), bovine erythrocytes carbonic anhydrase (30 k), aldolase (40 k), chicken egg white ovalbumin (45 k), catalase (56 k), bovine serum albumin (66 k), and phosphorylase b (97 k).

2.8. Texture profile analysis

After 150 min of cooling, cylinders of crumb (diameter 30 mm) were removed with a sharp borer from the three most inner slices (thickness 25 mm) of three bread replicates. The resultant nine cylindrical bread crumb samples were subjected to texture profile analysis (TPA) using an Instron (Norwood, MA, USA) 3342 device essentially as described by Bourne (1978). Crumb cylinders were compressed twice to 50% of their original height with a cylindrical probe (diameter 75 mm) at 100 mm/min with a 50 N load cell. Firmness (peak force during first compression), cohesiveness (ratio of force area during second compression to force area during first compression), resilience (ratio of force area down-stroke to force area up-stroke of the first compression), springiness (ratio of time needed for the second compression to that needed for the first compression) and chewiness (product of springiness, cohesiveness and hardness) were calculated from the TPA profiles.

2.9. Differential scanning calorimetry

To investigate the progress of starch gelatinization as a function of temperature, the portion of non-gelatinized starch crystals was determined in heat treated dough (15 min; 30 °C - 95 °C) with differential scanning calorimetry (DSC). DSC analyses were performed with a Q1000 DSC (TA Instruments, New Castle, DE, USA) calibrated with indium. Melting enthalpies were calculated using TA Instruments Universal Analysis software. An empty DSC pan served as reference. Freeze dried and milled samples were accurately weighed (2.5 – 4.0 mg) in aluminum pans (Perkin Elmer, Waltham, MA, USA), deionized water [1:3 (w/w) sample dm:water] was added, pans were hermetically sealed and after equilibration at 0 °C heated at 4 °C/min to 120 °C.

2.10. Statistical analysis

All experiments were performed at least in triplicate with statistical analysis (Student T-tests) at a significance level of 95% with JMP Pro 11.2.0 (SAS institute, Cary, NC, USA).

3. Results and discussion

3.1. Temperature dependence of Aq1 activity in the absence and presence of wheat flour extract

Under the above stated assay conditions Aq1 activity was optimal at 65 °C (**Figure 1**). Matsuzawa et al. (1988) noted optimal Aq1 activity at 70 °C on casein at pH 10.4. We here noted a decrease in Aq1 activity at temperatures exceeding 65 °C. Little if any activity remained at about 90 °C, in line with findings by Matsuzawa et al. (1988).

Use of 0.6 µl WFE in the Aq1 (1.0 U) activity assay resulted in 37%, 36%, 32% or 28% lower activities when incubated at 30 °C, 40 °C, 50 °C or 60 °C, respectively. Higher concentrations of

211 WFE lowered the Aq1 activity even more as the losses in hydrolytic activity at 50 °C were about
212 87% or 100% in the presence of 1.5 µl or 5.0 µl WFE, respectively. Wheat flour contains different
213 inhibitors of serine peptidases and, more specifically, of subtilisin (Di Maro, et al., 2011; Mundy,
214 Hejgaard, & Svendsen, 1984; Odani, Koide, & Ono, 1986; Østergaard, Rasmussen, Roberts, &
215 Hejgaard, 2000; Poerio, Di Gennaro, Di Maro, Farisei, Ferranti, & Parente, 2003). Some of the
216 latter may inhibit Aq1 since, as noted above, it is a member of the subfamily of the subtilisin-like
217 peptidases (Matsuzawa, et al., 1988).

218 The loss of peptidase activity when adding 0.6 µl WFE was lower at 70 °C and 75 °C (about 11%
219 and 10% respectively) than at 50 °C (about 32%). At temperatures of at least 80 °C, all inhibition
220 was lost. When higher levels of WFE were used, incubation at higher temperatures was necessary
221 to decrease the inhibition. When adding 5 µl or 25 µl WFE, inhibition only started to decrease
222 when the temperature had increased to 70 °C or 80 °C, respectively. With 5.0 µl WFE, 83% and
223 34% losses in peptidase activity were measured at respectively 70 °C and 75 °C.

224 The above allow concluding that inhibition of Aq1 by wheat endogenous inhibitors is temperature
225 dependent. We speculate that the loss of inhibition activity is caused by dissociation of the Aq1-
226 inhibitor complex, denaturation of the inhibitors or a combination of both. Apparently, inhibitor
227 concentration impacts the temperature at which Aq1 is no longer fully inhibited. It has been
228 documented that some serine peptidase inhibitors are inactivated at higher temperature (75 °C
229 - 95 °C). For example, barley alpha-amylase subtilisin inhibitor [BASI, Swiss-Prot P07596.2; 90.5%
230 similarity in amino acid sequence with wheat alpha-amylase subtilisin inhibitor, WASI, Swiss-Prot
231 P16347.1; clustal omega (European Bioinformatics Institute)] loses more than half or even all of

its inhibitory capacity after 10 min heat-treatment at 80 °C or 100 °C respectively (measured at pH 10.0) (Yoshikawa, Iwasaki, Fujii, & Oogaki, 1976). Another important serine peptidase inhibitor population in wheat flour is that of serpins (Rosenkrands, Hejgaard, Rasmussen, & Bjorn, 1994). For example, Tk-serpin [GenBank BAD85971.1; 31.2% similarity in amino acid sequence with serpins present in wheat, GenBank CAB52709.1, Clustal Omega (European Bioinformatics Institute)] inhibits subtilisin proteinase K [43% homology with Aq1 (Kwon, Terada, Matsuzawa, & Ohta, 1988)] and thermophilic Tk-subtilisin up to 100 °C (Tanaka, Koga, Takano, & Kanaya, 2011). Only a fraction serpin inhibitors in wheat flour are extractable with salt solution (Rosenkrands, Hejgaard, Rasmussen, & Bjorn, 1994). In our work, aqueous extraction of gluten did not recover all Aq1 inhibitors (results not shown). Complex formation and dissociation between Aq1 and its inhibitors and the impact of temperature thereupon evidently deserve further investigation.

3.2. Impact of Aqualysin 1 on the gluten network in bread

Aliquots of fermented dough (from recipes with and without Aq1) were heated for 15 min at different temperatures (30 °C – 95 °C). Next, all proteins were extracted under reducing conditions and subjected to SE-HPLC analysis. In **Figure 2.A**, the MW profiles of the eluates were divided into high MW (HMW-P, MW exceeding 18.4 k) and low MW (LMW-P, MW lower than 18.4 k) proteins. This rather arbitrary way of distinguishing between the two different protein classes was chosen because efforts to deconvolute the peaks were unsuccessful. Most GS and gliadins are part of the HMW-P, while at least a significant fraction of albumins and globulins is present in the LMW-P. HMW-P and LMW-P made up about 74% and 26% respectively of the total area of the protein extracted from dough which did not contain Aq1. That the SE-HPLC profiles of

the SDS-EP from mixed or fermented dough prepared from the Aq1 containing recipe were very similar to each other (results not shown) and to those of the corresponding control samples (**Figure 2.B**) indicates that Aq1 is not active during these unit operations. In addition, no shift in SDS-EP from HMW-P to LMW-P was noticed as a result of heating from 50 °C to 75 °C (**Figure 3**). The lack of Aq1 activity during mixing, fermentation and heat-treatments up to 75 °C was expected since the ratio of the levels of inhibitor to that of Aq1 was estimated to be 15 times higher in bread making than that when using the highest concentrations of WFE in the in vitro assay. However, when an aliquot of Aq1 containing dough was heated at temperatures exceeding 80 °C, significant losses of HMW-P occurred. HMW-P indeed decreased by 5% ($\pm 1\%$), 12% ($\pm 1\%$), 20% ($\pm 1\%$) and 29% ($\pm 1\%$) as a result of heating at 80 °C, 85 °C, 90 °C and 95 °C, respectively, and the losses were compensated by increases in extractable LMW-P. The clear shift in MW distribution indicates that partial hydrolysis of gluten proteins started around 80 °C during bread making. Aq1 probably became active again after dissociation from and/or inactivation of its inhibitor(s). In activity measurements with *p*-nitroanilide substrate, Aq1 was optimally active at 65 °C (**Figure 1**). When in the assay WFE (25 μ l) was added, the highest activity was measured at 85 °C, while in this bread-like system the highest Aq1 activity was noticed at about 95 °C (largest decrease in HMW-P levels, **Figure 3**). In contrast, no Aq1 activity was measured at temperatures exceeding 90 °C under the in vitro assay conditions. As a result of heating control dough for 15 min at 95 °C, 27% ($\pm 1\%$) of the total SDS-EP was extractable under reducing conditions as LMW-P. When the same experiment was done with Aq1 containing dough, the result was 56% ($\pm 1\%$) of the total SDS-EP level. Furthermore, in the standard bread making recipe (13 min at

274 232 °C), the SDS-EP level of LMW-P increased from 26% ($\pm 1\%$) (without Aq1) to 67% ($\pm 5\%$) when
275 Aq1 was included.

276 Protein extractions were also performed under non-reducing conditions. Due to cross-linking of
277 gluten proteins during baking, the level of SDS-EP decreased from 90% ($\pm 4\%$) in control dough
278 to 24% ($\pm 1\%$), in line with results of Lagrain et al. (2005). In the case of the Aq1 containing recipe,
279 the corresponding SDS-EP values were 91% ($\pm 3\%$) and 43% ($\pm 1\%$) respectively (**Figure 2.B**). That
280 the protein extractability under non-reducing conditions was 19% higher as a result of Aq1 use
281 points to the presence of hydrolysed fragments in the protein network of the former and thus to
282 a less coherent gluten network.

283 To better understand the impact of Aq1 during heat-treatment on gluten properties, the viscosity
284 of gluten suspensions was monitored while executing a temperature-time profile both in its
285 absence and in its presence (**Figure 4**). The viscosity of gluten suspensions decreased when the
286 temperature increased from 40 °C up to 95 °C. This is comparable to the findings of Lagrain et al.
287 (2005). None of the levels of Aq1 tested changed the viscosity profile significantly during this
288 heating step (Figure 4). Commercial gluten also contained Aq1 inhibitors (enzyme activity results
289 not shown). The latter postponed Aq1 activity during this first heating stage resulting in
290 insufficient gluten hydrolysis to change the viscosity. The viscosity increased during the holding
291 phase at 95 °C (Figure 4). This can be attributed to crosslinking of gluten proteins mainly by SH-
292 SS-exchange reactions (Lagrain, Thewissen, Brijs, & Delcour, 2008). Lagrain et al. (2005) noted a
293 steep viscosity increase when almost all glutenin and more than half of the gliadin proteins are
294 covalently incorporated in the gluten network. The increase in viscosity during the holding phase

at 95 °C was less pronounced when increasing concentrations of Aq1 were used (Figure 4). At the end of the holding phase, the maximal peak viscosity was significantly lower when higher concentrations of Aq1 were used. Aq1 activity during this heat-treatment resulted in a weakened gluten network.

Combined, the above RVA and protein extractability data allow concluding that Aq1 weakens and reduces the coherence of the gluten network during heating. Even though Aq1 is not active at temperatures below 80 °C, it can still significantly impact the gluten network formed as a result of bread making. As Aq1 becomes active during the later stages of bread baking, the relative contribution of post structure setting changes in the gluten network on the texture and structure of bread can be investigated.

3.3. Impact of Aq1 on the texture of fresh bread crumb

Inclusion of Aq1 in the standard bread making recipe impacted neither the volume of the resulting breads (Table 1) nor did it cause visually observable differences in crumb structure (Figure 5.A). However, it lowered crumb firmness, cohesiveness, springiness, resilience and chewiness significantly (Table 1). Also, the crumb disintegrated more easily. The large discrepancy between the significant impact on the gluten MW distribution and protein extractability described in Section 3.2 and the rather small changes in texture properties suggest a major role for starch in bringing about bread crumb texture.

DSC analysis showed that amylopectin in dough starts to melt at about 60 °C and that almost all crystals had melted when the temperature reached 85 °C (Figure 3). Addition of Aql had no impact on the amylopectin melting temperature (results not shown). The enzyme became active

after starch gelatinization started and is presumably active in the temperature range wherein significant gluten protein crosslinking occurs. A. P. Singh et al. (2005) have suggested that starch gelatinization stiffens the walls of the gas cells and limits gas cell expansion which subsequently impacts bread loaf volume. As starch gelatinization controls the end point for gas cell expansion, it can also influence crumb texture. Furthermore, it transforms visco-elastic dough into elastic crumb (Lagrain, Leman, Goesaert, & Delcour, 2008; Rouillé, Chiron, Colonna, Della Valle, & Lourdin, 2010; A. P. Singh & Bhattacharya, 2005).

When peptidases are active prior to starch gelatinization, gas cells can in some instances expand better than in control bread making resulting in larger volume and softer crumb (Caballero, Gomez, & Rosell, 2007; Indrani, Prabhasankar, & Venkateswara Rao, 2003). Since Aq1 only became active after both starch gelatinization and the transition of dough foam into crumb, it did not impact the gas cell expansion. Therefore, no changes in specific volume were noted. Only small changes in texture were noted which points to the importance of the starch matrix for bread texture. Further evidence that starch is an important structure determining component is that application of thermostable *Bacillus subtilis* alpha-amylase and *Bacillus stearothermophilus* maltogenic amylase in bread making largely impact the macroscopic crumb structure and initial crumb firmness, respectively (Lagrain, Leman, Goesaert, & Delcour, 2008).

3.4. Baking of model systems

The impact of gluten crosslinking and starch gelatinization on crumb structure was further investigated by preparing two unfermented dough model systems containing gluten and water (GD) or gluten, starch and water (GSD) with and without addition of Aq1. GD expanded

337 enormously during baking but collapsed upon cooling. Irregular shaped GD “breads” were
338 formed (**Figure 5.B**). In these model systems, the expansion was mainly due to water
339 evaporation. GD containing Aq1 expanded to a smaller extent during baking than control GD.
340 Probably because the expansion was less pronounced for GD containing Aq1 than for control GD,
341 the former did not visually collapse during cooling. In the GD containing Aq1, the peptidase
342 activity during baking weakened the gluten network which limited gas cell expansion upon water
343 evaporation. Furthermore, addition of Aq1 caused the “crumb” of GD to be softer and more
344 crumbly than that of control GD. When starch was used along with gluten, model dough systems
345 with comparable volumes and shapes were obtained (**Figure 5.C**). The GSD system prepared with
346 Aq1 addition was slightly more crumbly and less coherent than its control counterpart. Starch
347 gelatinization in the control GSD prevented enormous expansions and collapse of the gas cells
348 resulting in less open crumb structures and regular shapes. Starch gelatinization minimized the
349 impact of gluten network formation on the volume and structure of these model systems.
350 Nevertheless, gluten seemed important for the coherence of the final product as Aq1 use
351 increased the crumbliness of the baked product.

352 Gluten protein hydrolysis at temperatures exceeding 80 °C impacted texture only to a small
353 extent and had no impact on the specific volume or macroscopic appearance of bread crumb
354 (**Table 1** and **Figure 5.C**). Whereas gluten hydrolysis prior to starch gelatinization largely impacts
355 bread quality, its impact is less pronounced once the bread structure is set by gelatinized starch.
356 The main role of the thermoset gluten network seems to be its contribution to the coherence of
357 the final bread structure.

4. Conclusion

While proteins in WFE limit the activity of Aq1 at lower temperatures, they no longer do so at high temperatures. The mechanism(s) by which proteinaceous inhibitors impact Aq1 activity are temperature dependent. To the best of our knowledge, our report is the first to demonstrate the food application of a heat-reversible inhibition of a peptidase or, for that matter, an enzyme by proteinaceous inhibitors. Application of Aq1 in wheat bread making avoids gluten protein hydrolysis during mixing and fermentation and enables it at about 80 °C in the baking process. For the first time, it was possible to study the impact of heat-induced gluten network formation on the structure and texture of bread. Aq1 was indeed successfully used as a tool in bread making to study the impact of gluten network formation on bread crumb texture. Application of Aq1 had no impact on bread specific volume and only a rather small but still significant impact on the firmness of bread crumb. Aq1 peptidase activity during baking was responsible for a more crumbly and less coherent crumb structure than in control bread making. Of the physico-chemical changes during the baking phase, starch gelatinization is the main contributor to the overall bread texture and gluten polymerization seems to be important for the coherence of its crumb.

Acknowledgements

This work is part of the Methusalem programme 'Food for the Future' at KU Leuven. The authors thank Puratos (Groot-Bijgaarden) for financial support of the Peptibread project at KU Leuven. K. Brijs acknowledges the Industrial Research Fund (KU Leuven, Leuven, Belgium) for a position as

378 Industrial Research Manager. B. Vangeneugden and G. Gryp are thanked for technical assistance.

379 J. A. Delcour is W. K. Kellogg Chair in Cereal Science and Nutrition at KU Leuven.

380 *Note:*

381 Conflicts of interest: none

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Table 1: Texture profile data from double compression of bread crumb by 50% from control bread and bread produced from an aqualysin 1 (Aq1) containing recipe (control + Aq1). Values between brackets represent standard deviations. All listed parameters except the specific volumes were significantly lower for crumb from control + Aq1 than for crumb from control ($\alpha = 0.05$).

Figure 1: The decrease in level of *p*-nitroaniline released from N-succinyl-ala-ala-pro-phe-*p*-nitroanilide by aqualysin 1 with increasing concentrations of wheat flour extract (WFE) (0.0 μ l, 0.6 μ l, 1.5 μ l, 5.0 μ l and 25.0 μ l, areas indicated by grey-scales starting from white to dark-grey) for various incubation temperatures. Standard deviations are given with error bars.

Figure 2: SE-HPLC profiles of proteins extracted from control bread crumb (black line) and from bread crumb produced from an aqualysin 1 (Aq1) containing recipe (grey line) under reducing conditions (A). Eluted proteins were rather arbitrarily divided into high MW (HMW-P, MW > 18.4 k) and low MW (LMW-P, MW < 18.4 k).

SE-HPLC profiles of proteins extracted from fermented dough samples (dotted lines) and breads (full lines) from control (black lines) and an Aq1 containing recipe (grey) under non-reducing conditions (B). The most abundant gluten protein fractions present in the different peaks are indicated.

Figure 3: Levels of high molecular weight proteins [HMW-P, molecular weight (MW) > 18.4 k, dark grey] and low molecular weight proteins (LMW-P, MW < 18.4 k, light grey) extractable in

SDS containing medium under reducing conditions from unheated and heat-treated (15 min, 50 to 95 °C range) pieces of fermented aqualysin 1 (Aq1) dough (stacked columns). The scatter line indicates the percentages of gelatinized starch in the same samples.

Figure 4: Rapid visco analyzer profiles (grey) of gluten suspensions in water [17% (w/w) on protein basis, total weight of 25.00 g] without aqualysin 1 (Aq1) (full line) or addition of 47 U (dotted line) or 94 U (striped line) Aq1, subjected to a specific temperature-time profile (black).

Figure 5: Control and aqualysin 1 (Aq1) containing (control + Aq1) model bread systems consisting of bread loaves made according to a straight-dough method (A), 100% wheat gluten (B) or 25% wheat gluten and 75% commercial starch (C).