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ORIGINAL ARTICLE

Cross-linking of wheat gluten proteins during production of hard pretzels

Ine Rombouts · Bert Lagrain · Kristof Brijs · Jan A. Delcour

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Abstract The impact of the hot alkaline dip, prior to pretzel-baking, on the types and levels of cross-links between wheat proteins was studied. Protein extractability of pretzel dough in sodium dodecyl sulfate containing buffer decreased during alkaline dipping [45 s, 1.0% (w/v) NaOH, 90°C], and even more during baking (3 min at 250°C) and drying (10 min at 135°C). Reducing agent increased the extractability partly, indicating that both reducible (disulfide, SS) and non-reducible (non-SS) protein cross-links had been formed. The decrease in cystine levels suggested β-elimination of cystine releasing Cys and dehydroalanine (DHA). Subsequent reaction of DHA with Lys and Cys, induced the unusual and potentially cross-linking amino acids lysinoalanine (LAL) and lanthionine (LAN), respectively, in alkaline dipped dough (7 μmol LAN/g protein) and in the end product (9 μmol LAL and 50 μmol LAN/g protein). The baking/drying step increased sample redness, decreased Lys levels more than expected based on LAL formation (57 μmol/g protein), and induced a loss of reducing sugars (99 μmol/g protein), which suggested the potential contribution of Maillard-derived cross-links to the observed extractability loss. However, levels of Maillard products which possibly cross-link proteins, are small compared to DHA-derived cross-links. Higher dipping temperatures, longer dipping times, and higher NaOH concentrations increased protein extractability losses and redness, as well as LAL and LAN levels in the end product. No indications for Maillard-derived cross-links or LAL in pretzel dough immediately after dipping were found, even when severe dipping conditions were used.

Keywords Gluten · Beta-elimination · Dehydroalanine · Lysinoalanine · Lanthionine

Abbreviations

DHA	Dehydroalanine
HPAEC-IPAD	High-performance anion-exchange chromatography with integrated pulsed amperometric detection
LAL	Lysinoalanine
LAN	Lanthionine
mc	Moisture content
SDS	Sodium dodecyl sulfate
SE-HPLC	Size-exclusion high-performance liquid chromatography
SH	Sulfhydryl
SS	Disulfide

Introduction

Hard pretzels are popular savory wheat-based snacks, often with the shape of a knot or a stick. The dough, which typically consists of wheat flour, water, shortening, salt and a leavening agent, is shaped by relatively low pressure extrusion, treated with hot alkaline solution, and baked. Baking is divided into a rapid initial bake at high temperature, followed by a slow drying process at lower temperature (Delcour and Hoseney 2010; Walsh 1993; Seetharaman et al. 2004). The unique taste and hard shiny surface of pretzels result from the alkaline dipping prior to

I. Rombouts (✉) · B. Lagrain · K. Brijs · J. A. Delcour
Laboratory of Food Chemistry and Biochemistry and Leuven Food Science and Nutrition Research Centre (LFoRCe), Katholieke Universiteit Leuven, Kasteelpark Arenberg 20, P.O. Box 2463, 3001 Heverlee, Belgium
e-mail: Ine.Rombouts@biw.kuleuven.be

baking, typically for 30–45 s in a 1.0% (w/v) NaOH at 80–90°C. This treatment gelatinizes starch granules at the dough surface, dissociates amylose–lipid complexes, decreases levels of reducing sugars, and induces Maillard and caramelization reactions (Yao et al. 2006; Walsh 1993). Although the impact of such alkaline dip on the starch fraction is relatively well understood, the impact on the protein fraction is not clear. While conditions during pretzel production induce protein hydrolysis (Yao et al. 2006), it remains to be elucidated whether and/or to what extent they affect amino acid degradation and protein cross-linking. This study focuses on the formation of covalent protein cross-links, which has been already shown to greatly determine the texture and structure of various wheat-based foods (Lindsay and Skerritt 1999; Kuktaite et al. 2004) including bread (Payne et al. 1987) and pasta (Cubadda et al. 2007).

Gluten is the complex heterogeneous mixture of wheat storage proteins. It consists of monomeric gliadins and polymeric glutenins. Heat-induced gluten cross-linking is mainly ascribed to the formation of cysteine disulfide (SS) cross-links (Schofield et al. 1983), which are favored by high pressure and temperature (Kieffer et al. 2007), and alkaline pH (Visschers and de Jongh 2005). Indeed, SS cross-linking results from oxidation of reactive sulfhydryl (SH) groups and interchange reactions between SH groups and SS bonds (Lagrain et al. 2008). The reactivity of SH groups, which have a pK_a of 8.35 when fully exposed to water (Belitz et al. 2009a), is pronounced at alkaline pH, hence our interest is in evaluating the impact of dipping conditions on formation of SS cross-links in pretzels. Previous research using gluten model systems has suggested that the severe heat/alkali conditions during pretzel dipping may well induce β-elimination of cystine (Rombouts et al. 2010; Lagrain et al. 2010). In such reactions, the hydrogen atom of the chiral carbon of an intra- or intermolecular SS bond is first abstracted, and a persulfide in β-position of the chiral carbon is eliminated, yielding the amino acid dehydroalanine (DHA). Then, elimination of sulfur from the newly formed persulfide leads to Cys containing a free SH group, which can initiate SH oxidation or SH–SS interchange reactions. This way, SS cross-links can be formed, but DHA residues can also react with e.g. Cys, Lys, or His to respectively form the cross-links lanthionine (LAN), lysinoalanine (LAL), or histidinoalanine. An important distinction between SS and DHA-derived cross-links is that only the first are reducible. Although cross-linking as a result of β-elimination reactions is important for wool (Horn et al. 1941) and other (Linetsky et al. 2004; Zimmermann et al. 1993) proteins, literature about such cross-linking in food products is scarce and mainly limited to LAL formation (Maga 1984; Friedman 1999b; Sternberg et al. 1975). Not only cystine, but also Ser and Thr can undergo β-elimination, yet these amino acids are lost less rapidly (Whitaker and Feeney 1983). Furthermore, pretzel-making conditions seem favorable for a number of consecutive reactions classified under the term 'Maillard reaction' (Yao et al. 2006), that start with reactions between reducing sugars on the one hand and NH_2 groups of proteins, peptides or amino acids on the other. High temperature, low moisture content (mc), and alkaline conditions all promote these reactions, which inter alia involve the ε-NH_2 group of Lys (Belitz et al. 2009b). Maillard products have been mainly determined in food because of their impact on flavor, aroma, color and nutritional characteristics (Vaclavik and Christian 2008; Martins et al. 2000). However, in some cases, they cross-link two protein chains by bridging two amino acid side chains via a reducing sugar (Belitz et al. 2009b). Identified Maillard-derived protein cross-links include histidino-threosidine (Dai et al. 2007), pentosidine, bis-pyrraline, and bisarg (Belitz et al. 2009b).

The main objective of our work was to study the impact of the hot alkaline dip and subsequent baking on the formation of covalent cross-links between proteins at the surface of hard pretzels. More specifically, the importance of SS and non-SS cross-links will be compared, and the formation and relevance of DHA- and Maillard-derived cross-links will be evaluated. To that end, protein extractability, levels of possibly involved amino acids (Cys, Lys, Ser, Thr, His), pretzel color, and reducing sugar levels were monitored throughout the pretzel-making process. In addition, the impact of the dipping conditions (time, temperature, NaOH concentration) on protein cross-linking was studied.

Materials and methods

Materials

All solvents, chemicals and reagents were at least of analytical grade and purchased from Sigma-Aldrich (Steinheim, Germany) or VWR International (Leuven, Belgium), unless specified otherwise.

Commercial wheat flour [9.3% protein (N × 5.7) on dry matter basis, mc 11.2%] was from Meneba (Hasselt, Belgium), and commercial wheat gluten [74.7% protein (N × 5.7) on dry matter basis, mc 7.4%] was from Syral (Aalst, Belgium). Sugar was from Tiense Suikerraffinaderij (Tienen, Belgium), shortening (all vegetable, Crisco) was from J. M. Smucker (Orville, OH, USA), and compressed yeast was from Bruggeman (Ghent, Belgium).

Determination of protein and moisture contents

Protein contents were determined in triplicate, using an adaptation of the AOAC Official Method 990.03 (AOAC 1995) to an automated Dumas protein analysis system (EAS Variomax N/CN, Elt, Gouda, The Netherlands). A conversion factor of 5.7 was used to calculate protein from nitrogen content. The determination of mc was as described by AACC-I Approved Method 44-19 (AACC 2000).

Pretzel production

Flour (100.0 g), sugar (2.5 g), shortening (2.5 g), and yeast (0.5 g) were mixed for 3 min using a pin mixer (National Manufacturing, Lincoln, NE, USA). After addition of 50 ml water, the dough was mixed for 1 min, fermented for 20 min (30°C, 90% relative humidity), and sheeted (gap width 2.5 mm, National Manufacturing sheeter). Pretzel sticks [length 56.4 (±0.8) mm, height 2.6 (±0.1) mm, width 3.9 (±0.0) mm], were cut out of the dough. After 10 min fermentation, pretzel sticks were dipped for 15, 30, 45, or 60 s in 0.0, 0.5, 1.0, or 1.5% (w/v) NaOH at 50, 65, 80, or 90°C. The dipped pretzel sticks were baked for 3 min at 250°C, and dried for 10 min at 135°C. Samples were taken at three stages of the pretzel-making process: before dipping (control), after dipping, and after baking.

For all analyses except DHA determination, pretzels were prepared as described above. To evaluate potential DHA formation during dipping, gluten-water "dough" was prepared by manually mixing 100.0 g commercial gluten with 60 ml water. After a 20 min rest (30°C, 90% relative humidity), the gluten dough was rolled into balls (500 mg), which were dipped for 45 s in 1.0% (w/v) NaOH at 90°C. For DHA determination, the unheated gluten-water dough was taken as the control.

All samples were freeze dried and ground in a laboratory mill (250 µm, IKA, Staufen, Germany) prior to further analysis.

Determination of protein extractability

To determine the protein extractability in sodium dodecyl sulfate (SDS) containing media and the protein molecular weight distribution, size-exclusion high-performance liquid chromatography (SE-HPLC) was performed as described by Lagrain et al. (2005), using an LC-2010 system (Shimadzu, Kyoto, Japan) with automatic injection. Freeze dried samples [1.0 mg protein/ml] were extracted (60 min, 20°C) with a 50 mM sodium phosphate buffer (pH 6.8) containing 2.0% (w/v) SDS (Acros Organics, Geel, Belgium, which is further referred to as SDS buffer. The extractability of proteins in SDS containing media under reducing conditions was determined under nitrogen atmosphere. In this case, 2.0 M urea and 1.0% (w/v) dithiothreitol (Applichem, Darmstadt, Germany) were added to the SDS buffer. All analyses were performed in duplicate. After centrifugation (10 min, 11,000 *g*) and filtration over polyethersulfone (Millex-HP, 0.45 µm, Millipore, Carrigtwohill, Ireland), supernatants were loaded (60 µl) on a Biosep-SEC-S4000 column with separation range from 15,000 to 500,000 (Phenomenex, Torrance, CA, USA). The elution solvent was acetonitrile/water (1/1, v/v) containing 0.05% (v/v) trifluoroacetic acid. The flow rate was 1.0 ml/min and the column temperature 30°C. Protein elution was monitored at 214 nm. Extractability in SDS containing media (under non-reducing and reducing conditions) of any given sample was calculated from its peak area and expressed as percentage of total extractability. The latter was taken as the peak area of the unheated pretzel dough, extracted in SDS buffer under reducing conditions. It was assumed that (almost) all intermolecular SS bonds were cleaved during extraction under reducing conditions, based on the observation that the extractability of heavily cross-linked proteins did not increase by increasing the urea and DTT concentrations in the SDS buffer [up to 6.0 M, and 3.0% (w/v), respectively].

Determination of Lys, Ser, Thr, His, LAL, and LAN

Amino acids, potentially cross-linked, were first liberated by heating (24 h, 110°C) freeze dried samples (15.0 mg protein) in 1.0 ml 6.0 M HCl containing 0.1% phenol and 1.5 mM norleucine (as internal standard) after flushing the samples with nitrogen. Reaction mixtures were diluted (200-fold) in deionized water and filtered (Millex-GP, 0.22 µm, polyethersulfone, Millipore). Amino acids were then separated by high-performance anion-exchange chromatography with integrated pulsed amperometric detection (HPAEC-IPAD), using a Dionex BioLC system (Dionex, Sunnyvale, CA, USA) as described by Rombouts et al. (2009). Separation of 25 µl samples was performed at 30°C with an AminoPac PA10 guard (50 × 2 mm, Dionex) and analytical (250 × 2 mm, Dionex) column at a flow rate of 0.25 ml/min. Four eluents were used for the gradient mobile phases: water of at least 18.2 MΩ resistivity (A), and solutions of NaOH (B, 0.250 M, Mallinckrodt Baker, Deventer, The Netherlands), sodium acetate (C, 1.0 M, Dionex), and acetic acid (D, 0.100 M). Gradient conditions were as in Rombouts et al. (2009) and the detection waveform as in Ding et al. (2002). Amino acids, LAL (Bachem, Weil am Rhein, Germany), and LAN (TCI Europe, Zwijndrecht, Belgium) were detected using a gold working electrode and a pH reference electrode. Their levels were calculated using appropriate standards, and expressed on dry matter protein (µmol/g protein). All analyses were performed in triplicate.

Determination of Cys

Cys residues were first oxidized to Cys sulfonic acid and subsequently hydrolyzed and chromatographically quantified as described above. The oxidizing medium (3.0 ml, cooled to 0°C) contained 3.5% hydrogen peroxide and 90% formic acid, and was added to freeze dried sample (20.0 mg protein). The reaction mixture was stirred (15 min, 0°C) and then left overnight (16 h, 0°C). To reduce the excess of performic acid, 0.5 ml 48% hydrogen bromide was added, and the mixture was stirred for 30 min. The remaining bromine and formic acid were evaporated at 50°C, and samples were subjected to amino acid analysis.

Determination of DHA

Freeze dried samples of gluten-water dough (100 mg), either before or after the alkaline dip, were heated in sealed reaction tubes in 1.5 N HCl (0.50 ml) at 110°C for 120 min to liberate DHA as pyruvic acid, which was then determined colorimetrically after a clarification step (Rombouts et al. 2011).

Determination of total and reducing sugars

Levels of total and reducing sugars were determined by gas–liquid chromatography after acid hydrolysis and conversion into alditol acetates, as described by Cleemput et al. (1993), and Courtin et al. (2000), respectively.

Color determination

Average color parameters [L* (luminosity), a* (redness), b* (yellowness)] of ground samples were determined after five-fold measurement with a colorimeter (Colourquest 45/0 LAV, CQ/UNI-1600, HunterLab, Reston, VA,USA). Standard deviations of the average readings of three individual samples did not exceed 0.2.

Results and discussion

Disulfide cross-linking

Proteins of unheated pretzel dough, alkaline dipped (45 s, 90°C, 1.0% NaOH) pretzel dough, and baked pretzel were extracted in SDS containing buffer, separated based on molecular weight using SE-HPLC, and quantified (Fig. 1a). The main proteins in pretzel dough are gluten proteins. Under the used experimental conditions, polymeric glutenin and monomeric gliadin eluted before and after 7.2 min, respectively (Lagrain et al. 2005). The SE-HPLC profiles showed decreasing extractabilities of both fractions during alkaline dipping and baking. The glutenin proteins in baked pretzel were no longer extractable. The observed extractability loss in buffer which contains SDS, and hence limits if not nullifies all non-covalent interactions, can be explained by the formation of covalent cross-links. Literature emphasizes the importance of SS cross-links in and between gluten proteins, but non-SS cross-links may also contribute to the extractability loss. To distinguish between SS and non-SS cross-links, and to evaluate their separate impact on extractability loss, proteins were also extracted under reducing conditions, i.e. after cleavage of SS bonds (Fig. 1b). In what follows, extractabilities are reported as percentages of the total extractability of proteins in unheated pretzel dough under reducing conditions. In unheated pretzel dough, 79.4% of the proteins were extractable under non-reducing conditions. The alkaline dip reduced

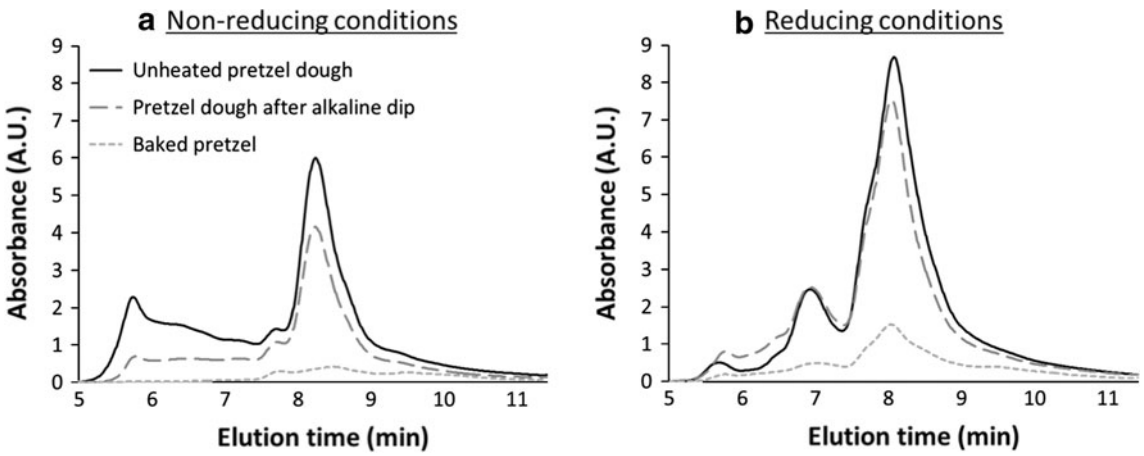

Fig. 1 SE-HPLC profiles of proteins in unheated pretzel dough, pretzel dough after alkaline dip [90°C, 45 s, 1.0% NaOH (w/v)], and baked pretzels. Proteins were extracted in SDS containing buffer under non-reducing (**a**) and reducing (**b**) conditions (see text). A.U.: arbitrary units

their extractabilities under non-reducing and reducing conditions to 46.6 and 92.5%, respectively, and baking reduced them further to 9.1 and 24.0%, respectively. The substantial increase of protein extractability upon reducing SS bonds illustrates the importance of SS cross-links, i.e. intermolecular cystine linkages, for gluten network formation. Based on the Cys level in pretzel dough, about 72 μmol/g SS cross-links could be formed. High temperatures and alkaline pH, such as during pretzel production, favor SH–SS interchange reactions, and hence the formation of SS cross-links (Lagrain et al. 2011). That reduction did not completely restore protein extractability of the dipped pretzel and the end product, illustrated that also non-SS cross-links were formed during dipping and baking.

DHA-derived cross-linking

Dipping decreased the Cys level from 144 μmol/g protein in the unheated dough to 134 μmol/g protein, and baking further decreased it to 51 μmol/g protein (Table 1). Decreasing Cys levels suggested that the alkaline conditions during pretzel-making induce β-elimination of cystine, possibly followed by the addition of Cys to the newly formed DHA, inducing LAN. Lys levels were not affected by dipping (45 s, 90°C, 1.0% NaOH), but were reduced by the subsequent baking step from 176 to 119 μmol/g protein in the final product (Table 1). Lys losses may be the result of LAL formation, Maillard reactions, and many other reactions which consume the reactive NH_2-group (Belitz et al. 2009a). Levels of Ser, Thr, and His did not significantly change (Table 1). However, due to the standard deviations on the results, it cannot be excluded that small amino acid losses have occurred.

It was difficult to determine DHA in pretzel dough, either unheated, after dipping, or after baking, probably due to interference of non-protein components during the colorimetric assay. A gluten-water "dough", which contains less non-protein components than a pretzel dough, was considered to be a good model system to evaluate DHA formation during pretzel-making. Dipping gluten-water dough for 45 s at 90°C in 1.0% NaOH resulted in a similar cystine loss as compared to the pretzel dough, and produced 4.0 μmol DHA/g protein. This supported the hypothesis that the alkaline conditions used for pretzel production induce β-elimination reactions. Subsequent baking did not alter the DHA level, suggesting that in this processing step the formation of DHA approximated its consumption by subsequent cross-linking reactions.

No LAL, but 7 μmol LAN/g protein was found in alkaline dipped pretzel dough. The final product contained 9 μmol/g protein LAL and 50 μmol LAN/g protein. Sternberg et al. (1975) detected 2 μmol LAL/g protein in pretzels. Unfortunately, they did not report on the applied pretzel-making conditions. Each mole of cystine yields one mole of DHA, which can react with Lys or Cys to form LAL or LAN, respectively (Friedman 1999a). Lagrain et al. (2010) found that these reactions do not alter the sum of the levels of cystine, DHA, LAN and LAL in gluten model systems, and this may well be valid during pretzel-making as well. However, the cystine loss (42 ± 3 μmol/g protein) during pretzel-baking did not correspond to the formation of LAN (43 ± 4 μmol/g protein) and LAL (9 ± 1 μmol/g protein). Although we did not observe Ser losses, it cannot be excluded that β-elimination of this amino acid occurred to a small extent, resulting in additional DHA formation. Unchanged Lys levels after dipping were in line with the absence of LAL in dipped dough, but the formation of 9 μmol LAL/g protein during baking only partly explained the loss of 57 μmol Lys/g protein.

Table 1 Levels of amino acids and sugars in unheated pretzel dough, after dipping for 45 s in 1.0% (w/v) NaOH at 90°C, and after subsequent baking (3 min at 250°C, 10 min at 135°C)

	Unheated	After alkaline dip	After baking
Cys (μmol/g protein)	144 ± 1	134 ± 6	51 ± 2
Lys (μmol/g protein)	176 ± 11	177 ± 16	119 ± 8
Ser (μmol/g protein)	435 ± 34	440 ± 31	438 ± 27
Thr (μmol/g protein)	219 ± 16	219 ± 6	215 ± 2
His (μmol/g protein)	173 ± 6	173 ± 4	179 ± 6
Total sugars (μmol/g dry matter)	5,373 ± 113	5,040 ± 123	4,938 ± 73
Reducing sugars (μmol/g dry matter)	150 ± 2	48 ± 7	33 ± 3

Maillard-derived cross-linking

Absolute levels of total and reducing sugars (Table 1) decreased during dipping and baking, probably as a result of the dispersion of starch and its hydrolysis derivatives into the dipping solution (Yao et al. 2006). The proportion of reducing-to-total sugars decreased from 2.8% in the unheated pretzel dough, to 1.0% after dipping, and 0.7% in the end product, which can be ascribed to several reactions, including Maillard. However, it should be kept in mind that under alkaline conditions carbohydrates are also susceptible to other reactions, such as degradation, isomerization, condensation, and polymerization (Belitz et al. 2009b). The Maillard reaction mainly yields orange and red colored products, and is thus correlated with increasing a* values on the Cielab color scale (Belitz et al. 2009b; Lamberts et al. 2008; Mestdagh et al. 2008). Dipping did not increase the a* value or Lys level, probably because high

humidity disfavors the Maillard reaction (Anese et al. 1999). In contrast, baking increased redness from −1.0 to 5.8, and led to disproportionate Lys losses (50 µmol/g protein). In this process step, a part of the reducing sugars, which decreased from 48 to 33 µmol/g protein, may well have reacted with Lys. Increasing a* values, decreasing reducing sugar levels, and disproportionate Lys losses during baking, support the hypothesis that the conditions during pretzel-making induce Maillard reactions, in agreement with Yao et al. (2006). However, the great impact of Maillard reactions on color, flavor, aroma, and nutritional quality of food, does not necessarily imply a great cross-linking potential. In fact, levels of Maillard-derived cross-links in pretzels are very small compared to those of DHA-derived cross-links. For instance, pretzels contain 0.03–0.06 µmol pentosidine/g protein (Henle et al. 1997).

Protein cross-linking during conventional pretzel-making: conclusions

Figure 2 gives an overview of reactions possibly contributing to protein cross-linking during pretzel-making. SE-HPLC results indicated that two types of covalent cross-links are formed during pretzel-making: reducible (SS) and non-reducible (non-SS) cross-links. SS cross-links are the result of the oxidation of free SH groups or SH–SS interchange reactions. Even though SS cross-links play a key role for the gluten network in pretzels, the presence of non-SS cross-links cannot be disregarded. Based on decreasing cystine levels, the potential formation of non-reducible DHA-derived cross-links after β-elimination of cystine was investigated. One such cross-link, LAN, was detected in pretzel dough after dipping, while another, LAL, was only found in the end products. A distinction should be made

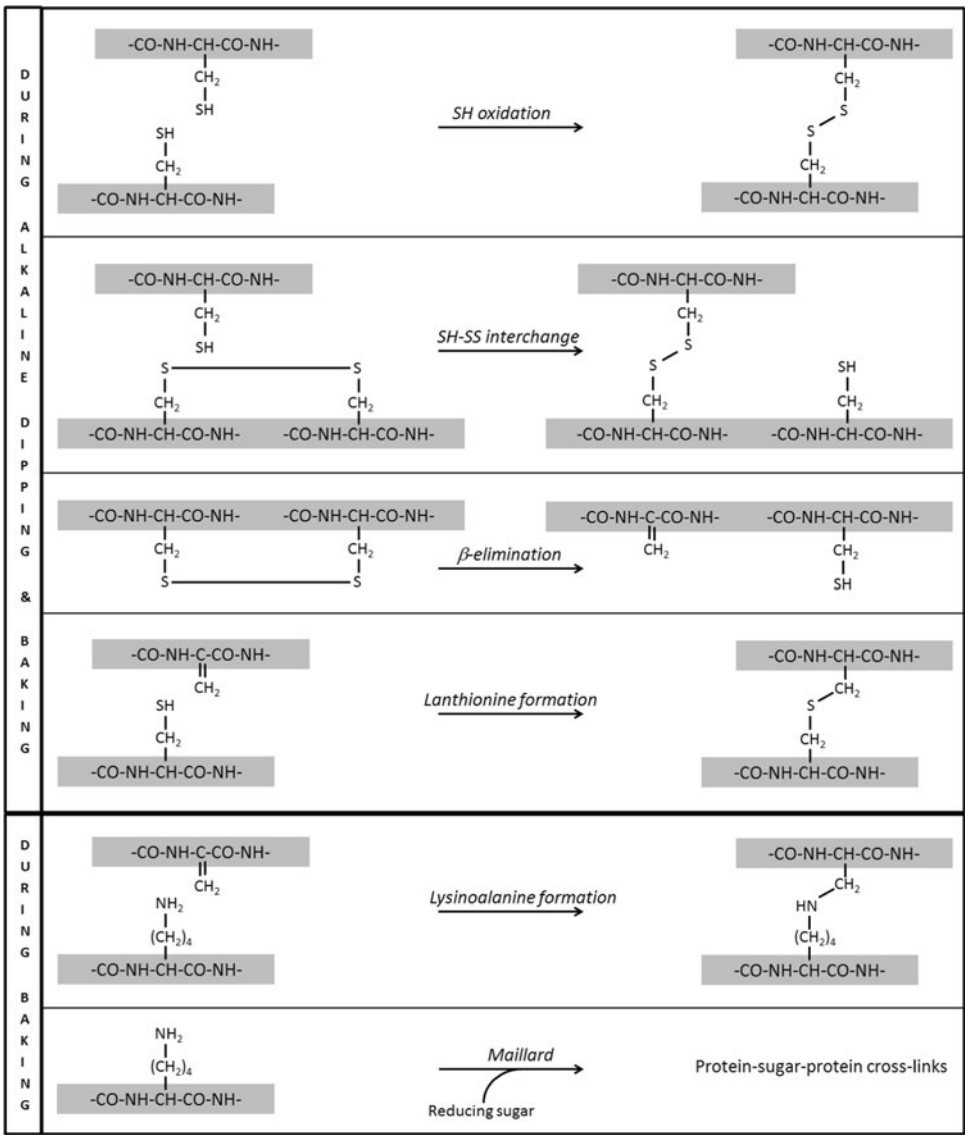

Fig. 2 Overview of reactions during pretzel-making resulting in protein cross-links

between intra-and intermolecular LAN and LAL. Friedman (1999a) concluded that intra- rather than intermolecular cross-links are formed in gluten, because protein concentration did not affect LAL formation. However, the introduction of intramolecular cross-links leaves the molecular weight of the treated protein largely unchanged, whereas the molecular weight increases proportionately with the number of intermolecular cross-links (Friedman 1999a). Hence, based on the observed increasing molecular weights of the reduced proteins during pretzel production, at least some LAN and LAL are assumed to form intermolecular links. Furthermore, disproportionate Lys losses during baking, together with decreasing reducing sugar levels, and increasing redness, suggested the occurrence of Maillard reactions in this process step. Yet, their importance for cross-linking could not be clearly established.

The impact of pretzel-making conditions on protein cross-linking

Reaction temperature, time, and pH impact cross-linking reactions in gluten (Lagrain et al. 2010; 2011). We here investigated the impact of deviations from the standard conditions on protein cross-linking during alkaline dipping. Pretzel dough was dipped for 45 s in 1.0% NaOH at 50, 65, 80, or 90°C. Firstly, no extractability loss was observed after dipping at 50°C (Fig. 3). Secondly, dipping at 65°C decreased the extractability under non-reducing conditions (Fig. 3a), but this could be restored by reduction (Fig. 3b), suggesting that mainly new SS cross-links were formed during dipping at lower temperature. Thirdly, dipping at 80 or 90°C decreased the extractability under non-reducing as well as reducing conditions, so higher temperatures induced SS as well as non-SS cross-link formation. The impact of dipping time on protein extractability was investigated by dipping pretzel dough for 15, 30, 45, or 60 s in 1.0% NaOH at 90°C. Protein extractability under non-reducing conditions decreased as a function of dipping time (Fig. 4a). Under reducing conditions (Fig. 4b) no decrease was noted during the first 15 s of the alkaline dip, which suggested that initially, mainly new SS cross-links caused the extractability loss. Longer dipping decreased the extractability as a function of dipping time, so after prolonged dipping also non-SS cross-links became detectable. Finally, dipping pretzel dough for 45 s in 0.5, 1.0, or 1.5% NaOH at 90°C decreased protein extractability under non-reducing conditions from 79.4 to 45.4%, and subsequent baking further reduced it to 9.1% (Fig. 5a), irrespective of the applied NaOH concentration. Under the experimental conditions, extractability loss under non-reducing conditions was apparently not impacted by NaOH concentration, possibly because the dipping solutions with 0.5, 1.0, and 1.5% (w/v) NaOH only had a slightly different pH, i.e. 13.1, 13.4, and 13.6, respectively. However, higher NaOH concentrations increased extractability losses during dipping under reducing conditions (Fig. 5b), indicating that they led to more non-SS cross-links.

In conclusion, alkaline dipping of pretzel dough for a longer time or at a higher temperature, leads to an increased extractability loss, due to increased protein cross-linking. SE-HPLC results only indicate the formation of SS cross-links during dipping under mild dipping conditions, while indications for both SS and non-SS cross-links were found after dipping for a longer time and at higher temperatures. Some of the non-SS cross-links formed during dipping may well be LAN, of which the level in the dipped pretzel dough increased with dipping temperature, time, and NaOH concentration (Fig. 6). No indications for non-

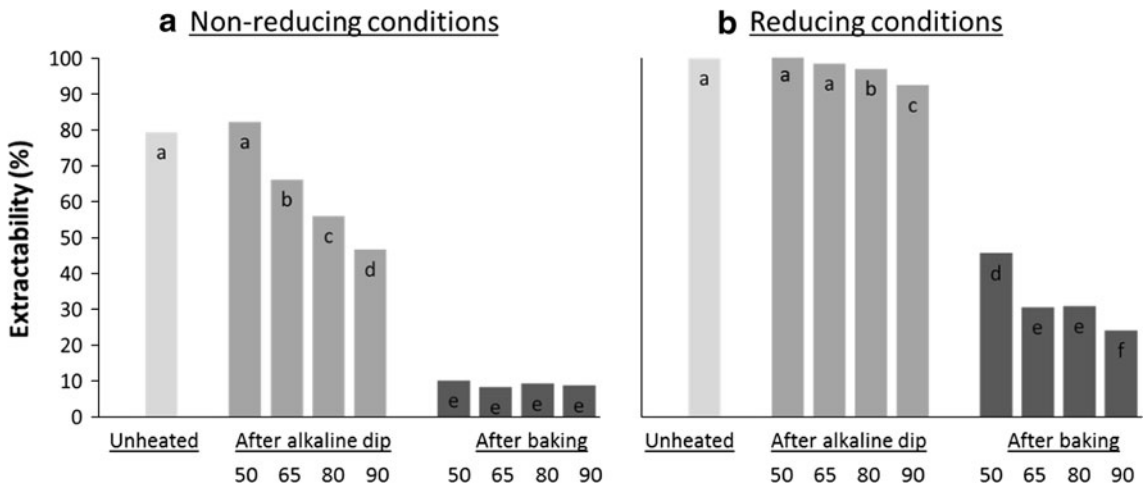

Fig. 3 Impact of dipping temperature. Protein extractabilities in SDS containing buffer under non-reducing (**a**) and reducing (**b**) conditions of unheated pretzel dough, pretzel dough after alkaline dip, and baked pretzels. Pretzel dough was dipped for 45 s in 1.0% (w/v) NaOH at 50, 65, 80 or 90°C. Extractabilities of samples indicated with the same letter are not significantly different ($P > 0.05$, $\alpha = 0.05$)

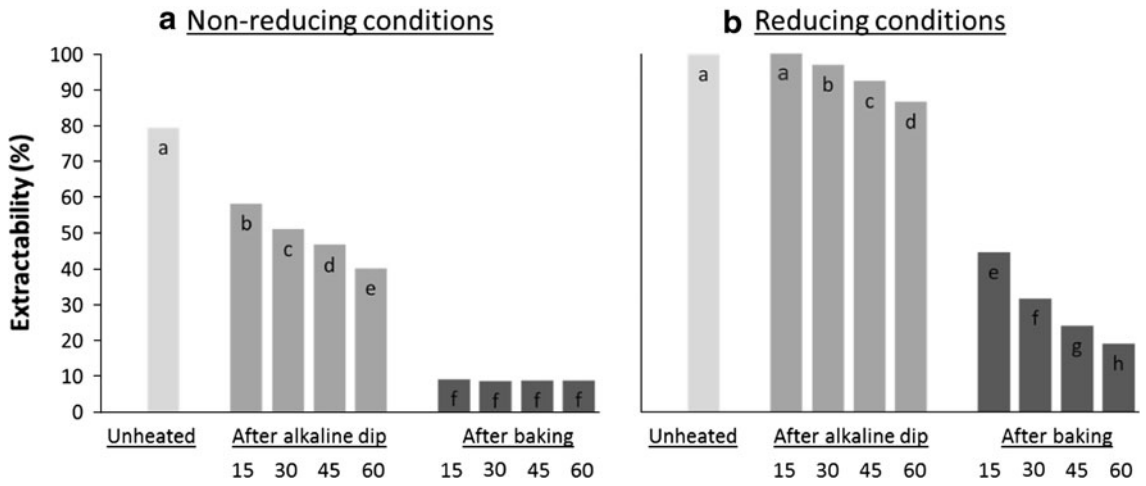

Fig. 4 Impact of dipping time. Protein extractabilities in SDS containing buffer under non-reducing (**a**) and reducing (**b**) conditions of unheated pretzel dough, pretzel dough after alkaline dip, and baked pretzels. Pretzel dough was dipped for 15, 30, 45, or 60 s in 1.0% (w/v) NaOH at 90°C. Extractabilities of samples indicated with the same letter are not significantly different ($P > 0.05$, $\alpha = 0.05$)

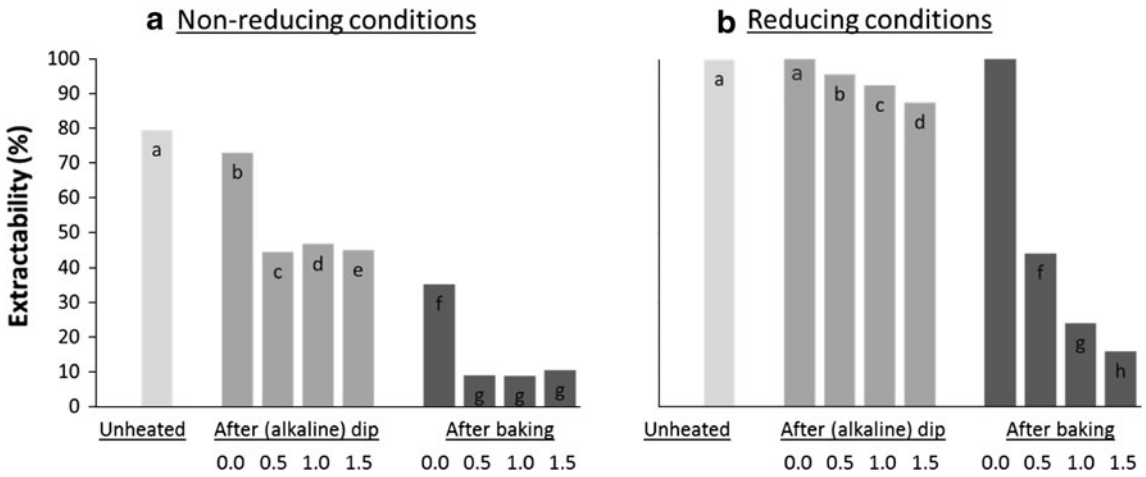

Fig. 5 Impact of NaOH concentration. Protein extractabilities in SDS containing buffer under non-reducing (**a**) and reducing (**b**) conditions of unheated pretzel dough, pretzel dough after (alkaline) dip, and baked pretzels. Pretzel dough was dipped for 45 s in 0.0, 0.5, 1.0, or 1.5% (w/v) NaOH at 90°C. Extractabilities of samples indicated with the same letter are not significantly different ($P > 0.05$, $\alpha = 0.05$)

SS cross-links other than LAN were found in any sample immediately after dipping. Indeed, even dipping for 60 s (90°C, 1.0% NaOH), or in 1.5% NaOH (45 s, 90°C), did not induce noticeable LAL, formation (Fig. 7). Neither did it increase a* values (results not shown). Again, it should be noted that LAL can be formed both intra- and intermolecularly.

Baking reduced the protein extractability under non-reducing conditions further to a minimum of 9.1%, irrespective of the applied dipping temperature, duration, or concentration (Figs. 3a, 4a, 5a). In contrast, the protein extractability loss under reducing conditions was larger when higher temperatures, longer dipping times, or higher NaOH concentrations were applied, probably due to more non-SS cross-link formation (Figs. 3b, 4b, 5b). More severe dipping conditions yielded pretzels with higher redness and higher LAN (Fig. 6) and LAL (Fig. 7) levels. Indeed, the formation of Maillard reaction products increases redness, and LAN and LAL are potential cross-links. The linear correlations between the extractability loss under reducing conditions and the sum of LAN and LAL levels ($r = 0.988$) supported the hypothesis that the formed LAN and LAL links are at least partly intermolecular, and hence contribute to the protein network. Maximum LAL levels (14 μmol/g protein) were found in the pretzels dipped for 45 s at 90°C in 1.5% NaOH, while LAN formation was maximal (52 μmol/g protein) after dipping for 60 s at 90°C in 1.0% NaOH. Another interesting observation was that even dipping at 50°C (45 s, 1.0% NaOH), or dipping in 0.5% NaOH (45 s, 90°C), or dipping for 15 s (90°C, 1.0% NaOH) led to end

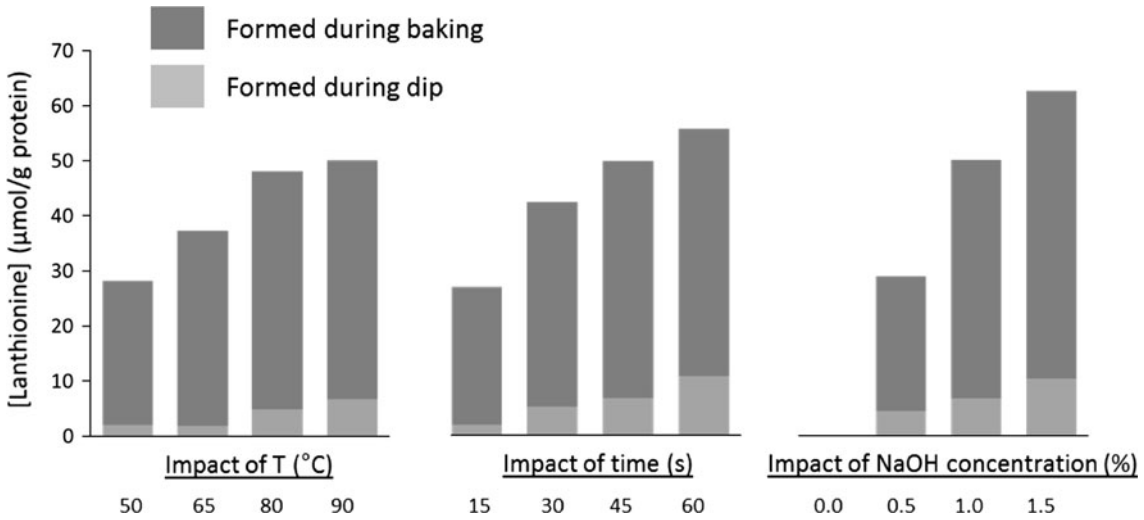

Fig. 6 Impact of temperature and concentration of NaOH, and duration of dipping, on lanthionine (LAN) levels (µmol/g protein) in pretzel dough after (alkaline) dip, and after baking. Pretzel dough was dipped for 45 s in 1.0% (w/v) NaOH at 90°C, unless specified otherwise

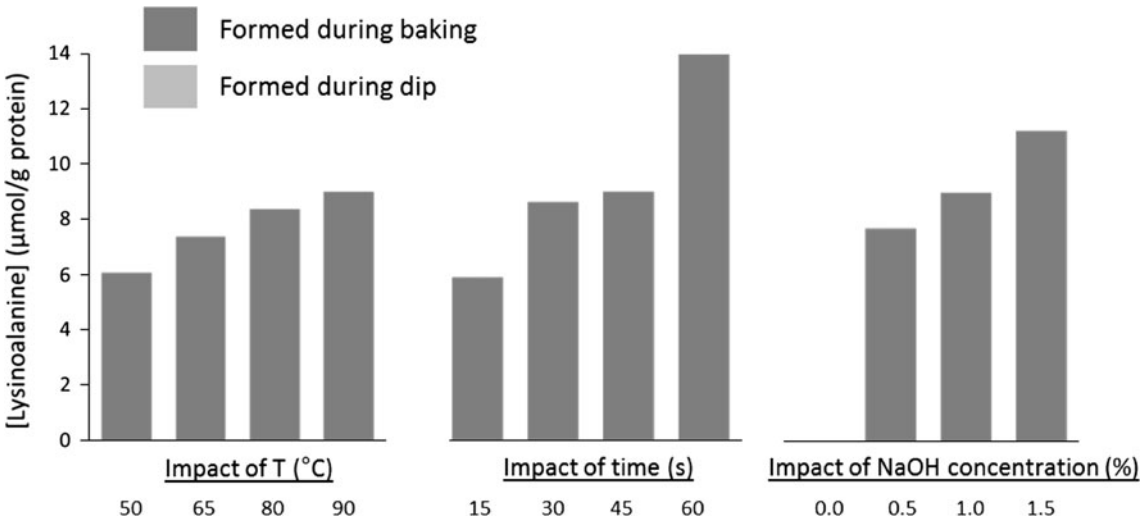

Fig. 7 Impact of temperature and concentration of NaOH, and duration of dipping, on lysinoalanine (LAL) levels (µmol/g protein) in pretzel dough after (alkaline) dip, and after baking. Pretzel dough was dipped for 45 s in 1.0% (w/v) NaOH at 90°C, unless specified otherwise

products of which the protein fraction was not completely extractable under reducing conditions, which demonstrated the importance of non-SS cross-links, *e.g.* LAN, LAL and Maillard-derived cross-links, into the protein network during pretzel dipping.

To further investigate the impact of pH, we evaluated protein cross-linking during dipping for 45 s in water at 90°C and subsequent baking and drying. The dip at neutral pH decreased protein extractability under non-reducing conditions from 79.4 to 72.9%, and subsequent baking further decreased it to 35.1% (Fig. 5a). This extractability loss was caused by formation of SS cross-links, as protein extractability under reducing conditions remained constant during the dip in water and subsequent baking (Fig. 5b). No LAN or LAL were found in the products obtained by dipping in water and subsequent baking (Figs. 6 and 7). Thus, in comparison to alkaline dipping, dipping in water limited SS cross-linking and even prevented non-SS cross-linking. These results suggest that the protein network in bagels, which are boiled in water prior to baking, is probably less strong than that in pretzels. The alkaline conditions during dipping make the pretzel-making process unique.

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