¹⁵N-Labeling of Egg Proteins for Studying Protein Network Formation During Pound Cake Making

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ABSTRACT

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Proteins from wheat and egg are important for pound cake texture, but their exact role is insufficiently understood. A clear, analytical distinction between proteins from wheat flour, egg white, or egg yolk has been a main challenge. However, this can be addressed by using egg proteins carrying ¹⁵N. Therefore, egg white and yolk protein were enriched in ¹⁵N by mixing ¹⁵N-labeled leucine into hen feed. Incorporation of egg and flour proteins in the protein network was monitored based on changes in their extractability during cake making. The relative contribution of different noncovalent and covalent bonds could be determined by using different extraction media. We

for the first time distinguished between the contribution of egg white, egg yolk, and wheat protein in network formation during pound cake making. Our results show that during batter mixing hardly any intermolecular disulfide bonds are formed and that baking induces tremendous changes in protein extractability. A protein network based on both disulfide bonds and hydrophobic interactions is formed during baking. This covalent network includes almost all egg white protein and most of the yolk and wheat flour protein. The remaining protein fraction most probably lacks sulfhydryl groups and/or intramolecular disulfide bonds.

Pound cake is made from equal amounts of fresh eggs, margarine or butter, sugar, and wheat flour. Typically, 45% of the proteins in the batter originate from wheat flour, 30% from egg white, and 25% from egg yolk (Wilderjans et al. 2013). Egg white makes up about two-thirds of the liquid egg mass. It consists of 10–11% protein, of which ovalbumin is the most important (54% of the egg white protein) and the only one containing free sulfhydryl (SH) groups. The other egg white proteins contain intramolecular disulfide (SS) bonds but no free SH groups (Powrie and Nakai 1985). Egg white gels as a result of denaturation of its proteins (Li-Chan et al. 1995). The four SH groups of ovalbumin are exposed during heating and can therefore react with other SH groups or with intramolecular SS bonds to form intermolecular SS bonds, which are crucial for the network of egg white gels (Beveridge and Arntfield 1979; Van der Plancken et al. 2005). The three-dimensional network consists of aggregates of denatured protein linked by hydrophobic, electrostatic, and covalent bonds (Ma and Holme 1982).

Egg yolk contains 16% protein. It is a dispersion of granules in liquid plasma that can be fractionated after dilution and centrifugation. Both granules and plasma contain about half of the egg yolk protein (Anton 2013). In contrast to most other yolk proteins, phosvitin (part of the granules) contains neither SH groups nor intramolecular SS bonds (Huopalahti et al. 2007). Plasma protein (livetin and vitellenin) dominates the gel-formation process. However, after disintegration, the granule components also contribute to network formation. The overall egg yolk gel is based on hydrophobic interactions and intermolecular SS bonds (Kiosseoglou and Paraskevopoulou 2005).

The extraction method of Osborne (1907) divides wheat flour protein into nongluten (15–20%) and gluten protein (80–85%). Nongluten protein contains the water-extractable albumin and the globulin, which are extractable in water and dilute salt solutions (Delcour et al. 2012). Gluten protein consists of gliadin and glutenin, which are extracted neither in water nor in salt solutions. Gliadin is mainly monomeric, extractable in aqueous ethanol, and

contains no free SH groups. There are three types of gliadin (α , γ , and ω), of which the α - and γ -gliadins contain intramolecular SS bonds. Glutenins are polymeric proteins that consist of high- and low-molecular-weight glutenin subunits. These subunits contain both free SH groups and intramolecular SS bonds (Veraverbeke and Delcour 2002). During dough mixing, SH/SS exchange reactions occur between glutenins. Because the intramolecular SS bonds of the different gliadins are stable at room temperature, they do not participate in these reactions (Delcour et al. 2012). However, during baking, SH oxidation reactions take place along with heat-induced SH/SS exchange reactions (Veraverbeke et al. 1999; Singh 2005). Gliadins (but not ω -gliadin) are then incorporated into the heat-induced SS network (Schofield et al. 1983).

It has been hypothesized that protein from egg white, egg yolk, and wheat flour form a gel network with intermolecular covalent SS bonds, which codetermines the structure of products such as cake (Kiosseoglou 2004). According to Kiosseoglou (2004), egg white protein may influence yolk gelation and, as such, the texture of the product. Earlier research from our group showed that the formed protein network is crucial for texture and volume of pound cake (Wilderjans et al. 2008, 2010b, 2010c; Deleu et al. 2015). More specifically, the egg white protein ovalbumin plays a key role in protein network formation. The timing of denaturation and incorporation of ovalbumin determines the incorporation of other proteins, which impacts cake springiness and cohesiveness (Deleu et al. 2015). However, there remains a large gap in knowledge about the formation of the protein network, the proteins involved, and the structure of the final network.

No studies have yet reported how to clearly, analytically distinguish between proteins from wheat flour, egg white, or egg yolk. However, the stable isotope ¹⁵N can be used as an intrinsic label of egg white and egg yolk protein. Stable isotope technology has proven its usefulness in fundamental biology and medicine (Gruhn and Richter 1977; Hobson 1995; Evenepoel et al. 1997) but is new in bakery applications. Several authors have enriched egg proteins with both ¹³C- and ¹⁵N-isotopes in different chicken breeds with different amino acids and with complementation (Evenepoel et al. 1997) or supplementation (Geboes et al. 2004; Fromentin et al. 2012) of the amino acids to the feed. Because transamination occurs during the metabolism (Gruhn and Hennig 1984), the proteins are rather uniformly labeled (Evenepoel et al. 1998; Fromentin et al. 2012).

To date, because there is no clear way to distinguish between different protein sources in batter, the insight into protein network formation during pound cake making is poor. The aim of this work was to introduce ¹⁵N-labeling of egg proteins as a tool for studying

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the protein network in cake. In a first experimental approach, an Osborne-type protein fractionation protocol was developed. In the next step, labeled egg white or egg yolk was interchanged with the unlabeled counterpart and used in pound cake making. After fractionation, the quantity and overall ^{15}N -enrichment of extracted protein were determined. Thus, the extractability of egg white, egg yolk, and wheat flour protein from batter and cake was mapped. This allowed estimating the relative contribution of each ingredient in the protein network formation during pound cake baking.

The presented protein fractionation protocol and ^{15}N -labeling of egg proteins were used in Deleu et al. (2016, 2017) for studying the incorporation of different protein types as a function of temperature during baking. The role of egg white proteins and wheat flour gliadins were further discussed in Deleu et al. (2016), and Deleu et al. (2017) mainly dealt with egg yolk and its different fractions in pound cake making.

MATERIALS AND METHODS

Materials. Eggs were collected from Isa Brown hens within 4 h after being laid (see the ^{15}N -Enrichment of the Egg Proteins section) and were used for batter preparation on the same day. This way, the conversion of ovalbumin to S-ovalbumin, which influences the protein network formation in pound cake (Deleu et al. 2015), was restricted. Wheat flour (14.0% moisture, 10.2% protein [as is basis]) was from Paniflower (Merksem, Belgium). AACC International Approved Method 44-15.02 was used to determine flour moisture content and an adaptation of the AOAC official method (AOAC 1995) to determine nitrogen content (conversion factor 5.70) with an automated Dumas protein analysis system (EAS VarioMax C/N, Elt, Gouda, the Netherlands). Margarine (19.3% moisture) was from Puratos (Groot-Bijgaarden, Belgium), and sodium bicarbonate and sodium acid pyrophosphate (SAPP 28) were from Budenheim (Budenheim, Germany). Sugar and chicken feed were purchased in a local supermarket. ^{15}N -L-leucine (98 atom percentage [AP],) was from Campro Scientific (Veenendaal, the Netherlands).

All reagents, solvents, and chemicals were of analytical grade and obtained from Sigma-Aldrich (Bornem, Belgium) unless indicated otherwise.

Preparation of Pound Cake and Batter Samples. Cake batter was made with fresh egg white (300.0 g), fresh egg yolk (150.0 g), wheat flour (450.0 g on 14% moisture base), sugar (450.0 g), margarine (450.0 g), water (80.0 g), and baking powder (4.82 g of sodium bicarbonate and 6.43 g of sodium acid pyrophosphate), resulting in an aqueous phase that made up 52% of the batter weight, with a sucrose concentration of 48% (sucrose weight/aqueous phase weight). A multistage mixing method was used and the batters baked in an electrical resistance oven as in the study by Luyts et al. (2013). The mixing process started with a creaming step (margarine and sugar), followed by addition of the eggs, and ended with addition of the flour and baking powder. The baking profile was mimicked from that in the center of a cake baked in a conventional oven, that is, from 25 to 100°C in 37 min 30 s and constant at 100°C for 17 min 30 s.

Samples were taken from the unbaked batter and from the final cake. They were immediately frozen with liquid nitrogen and freeze-dried. The freeze-dried samples were ground with a mortar and pestle. Samples (2.0 g) were defatted by shaking with 20.0 mL of hexane in a 50.0 mL glass test tube for 60 min. After removing

the hexane phase, the extraction was repeated a second time, and the resulting residues were air dried at ambient temperature. Cakes were baked in duplicate for both egg white and egg yolk labeling. One sample was taken from each batter and cake.

Protein Extractability. Aliquots of freeze-dried egg white (about 0.1 g), freeze-dried and defatted egg yolk (about 0.3 g), flour (about 0.8 g), and freeze-dried and defatted batter and cake (about 1.0 g) were weighed in centrifuge tubes to represent 100.0 mg of protein. The nitrogen-to-protein conversion factors used were 6.25 for egg, 5.70 for flour, and 6.00 for both batter and cake protein. Media A, B, C, and D (Table I) were added to the samples in that order, with removal of the resultant extracts in between. Each medium extraction (with 3.0 mL) was performed three times except for the first medium (A), for which 6.0 mL was added at once and an additional extraction with 3.0 mL was performed. Each time, the mixtures were vortexed, shaken (150 rpm, 10 min, 23°C; Laboshake LS500, Gerhardt, Königswinter, Germany) and centrifuged (12,000 $\times$ g, 10 min, 10°C; J2-21 centrifuge, Beckman, Fullerton, CA, U.S.A.). The resulting supernatants were decanted, pooled, filtered (0.45 μm , Millex-HP, Millipore, Darmstadt, Germany), and dried at 95°C for 10 h before determination of the protein content by Dumas analysis. The yield of extracted protein was calculated for each medium as the ratio of extracted to total protein in the initial sample. Additional extraction steps with the same medium did not yield more protein. Extractions were executed in triplicate for each sample with standard deviations for each extraction yield smaller than 4%.

Next to the above fractionation protocol, an extraction of the cake samples was executed with medium E (Table I). In this approach, different combinations of only media C, D, and E were used to determine the importance of both hydrophobic and SS bonds. These extractions were performed in duplicate.

^{15}N -Enrichment of the Egg Proteins. Isa Brown hens (32–35 weeks old) were kept in a closed, air-conditioned henhouse. Four of 25 hens were selected to consume ^{15}N -leucine-supplemented feed during 18 days. To that end, ^{15}N -leucine was mixed into normal chicken feed meal (3.0 g/kg). The eggs were collected daily, and the ^{15}N -enrichments of both egg white and yolk were measured on a freeze-dried sample with an elemental analyzer–isotope ratio mass spectrometer system (FlashHT with DeltaV Advantage, Thermo Fisher Scientific, Waltham, MA, U.S.A.). Calibration was with a combination of standards (acetanilide, IAEA-N1). This yielded the AP of the sample, which is the ratio of ^{15}N -isotopes to the total amount of nitrogen (equation 1):

$$\text{AP} = \frac{a_2^{15}\text{N}}{a_1^{14}\text{N} + a_2^{15}\text{N}} \quad (1)$$

where $a_1^{14}\text{N}$ and $a_2^{15}\text{N}$ are the amounts of ^{14}N and ^{15}N atoms, respectively.

The atom percentage excess (APE) is the AP of the sample corrected for the AP of the reference (i.e., atmospheric air, 0.366%) and reflects the ^{15}N -enrichment in a sample (Mariotti et al. 1981).

Use of ^{15}N -Labeled Egg Proteins in Pound Cake Batter. After 10 days of feeding hens with ^{15}N -leucine-supplemented feed, cake batter was made with labeled eggs, which were used within the first 6 h after being laid. First, labeled egg white was manually separated from labeled yolk. The labeled egg white was then used in a batter with unlabeled egg yolk and vice versa to obtain only either labeled egg white or labeled yolk protein. Labeled egg white (or egg yolk) was diluted with unlabeled fresh egg white (or egg yolk) from eggs of hens with the unlabeled diet for cost reasons. This was possible because the eggs from the four different chickens were highly enriched in the label. Labeled egg white was added to about the same quantity of its unlabeled counterpart. Likewise, labeled yolk was mixed with about twice the quantity of its unlabeled variant.

The overall enrichment of the batter protein is the sum of the pooled enrichments of labeled and unlabeled proteins originating

TABLE I
Media Used in the Extraction Protocol

Medium	Composition
A	0.40M NaCl in 0.10M sodium phosphate buffer (SPB) (pH 7.6)
B	60% ethanol (v/v)
C	2.0% (w/v) sodium dodecyl sulfate (SDS) in 0.10M SPB (pH 7.6)
D	2.0% (w/v) dithiothreitol (DTT) in 0.10M SPB (pH 7.6)
E	2.0% (w/v) SDS and 2.0% (w/v) DTT in 0.10M SPB (pH 7.6)

from egg white, yolk, and flour (equation 2). The APEs of egg white and yolk, but also those of the unlabeled ingredients and of the batter itself, can be determined. From these experimental data, the fraction (f) of the proteins labeled was calculated by using equation 2. Using this approach with only one source of ^{15}N -labeled protein allowed quantitation of the labeled protein in the extracts of both batter and cake:

$$\text{APE}_{\text{batter}} = [f_{\text{LP}} \times \text{APE}_{\text{LP}}] + [(1 - f_{\text{LP}}) \times \text{APE}_{\text{ULP}}] \quad (2)$$

where LP indicates labeled proteins and ULP indicates unlabeled proteins.

Combining the data for egg white, egg yolk, and the total extractability, the extractability of the unlabeled wheat flour protein was also mapped. Equation 2 also applies for the protein extracts (starting from the APE of the extract), being the pooled sum of different labeled and unlabeled proteins. This equation yields a specific quantification of the ingredient proteins in the different extracts. As a control, ^{15}N -analysis of the batters was used to calculate the relative contribution of each ingredient to the total batter protein population. This showed that egg white accounted for 31% of all batter protein, yolk for 24%, and flour for 45%. These values were in line with the theoretical values (respectively 30, 25, and 45%) and pointed to the reliability and quantitative character of the labeling method.

RESULTS AND DISCUSSION

Extractability of Protein from Batter, Cake, and Its Separate Ingredients. First, an Osborne-type protein fractionation protocol was developed. The use of water as a first extraction medium made no sense for experiments with cake batter, because it contains a high level of ions from the baking powder. We therefore did not distinguish between wheat albumin and globulin and extracted both with the salt-containing buffer (medium A). Next, gliadin was extracted with aqueous ethanol (medium B) from the wheat flour residue. Sodium dodecyl sulfate (SDS) has high ionic strength and breaks up hydrophobic bonds in and between proteins (Bhuyan 2010). SDS has been used in earlier studies on protein network formation. Changes in the level of extractable proteins during and after a heating process were a measure for protein network formation (Veraverbeke et al. 1999; Wilderjans et al. 2010a). The last medium contained dithiothreitol (DTT). DTT breaks SS bonds within proteins by SH/SS exchange reactions (Cleland 1964). It can therefore show the presence of an SS bond-based network (Jansens et al. 2011). A DTT-containing medium also releases the subunits from

glutenin and renders them extractable (Veraverbeke and Delcour 2002). With these four media, the extractability of the proteins from the individual ingredients (i.e., wheat flour, egg white, and egg yolk) was compared with those of the proteins from batter and cake.

Extractability of Protein from Separate Ingredients. Almost all egg white protein (94%) was extractable in medium A (Fig. 1), which is in line with the literature. Egg white mainly contains water-soluble proteins (Powrie and Nakai 1985). Ovomucin has a high molecular weight and is hardly soluble except when using SDS and reducing agents (Robinson and Monsey 1964; Abeyratne et al. 2013).

Most of the egg yolk protein (88%) was extracted in medium A (Fig. 1). The levels of protein extracted with media B, C, and D were 2, 4, and 5%, respectively (Fig. 1). Based on their stability in aqueous solutions, it is likely that the plasma proteins (livetins and vitellin) are part of fraction A. Also, because of the high ionic strength of medium A, the granules may have disintegrated during the extraction procedure. This way, the granule proteins phosvitin and vitellin could be extracted. Phosvitin has a large hydrophilic part and is soluble in water (Huopalahti et al. 2007). In conclusion, fraction A contains many different egg yolk proteins.

In the particular cake flour used, albumin and globulin together accounted for 19% of the protein (medium A) (Fig. 1) and gliadin extracted in aqueous ethanol (medium B) for 42%. The remaining flour protein was glutenin (Osborne 1907). Part of the latter (13% of the total flour protein) was extracted in medium C (Fig. 1), whereas the remaining glutenin fraction was extracted as subunits after breaking the intramolecular SS bonds in medium D (Fig. 1). Overall, the yields of the different wheat flour protein fractions were in line with literature data (Veraverbeke and Delcour 2002; Duyvejonck et al. 2011).

Extractability of Protein from Batter. The majority (56%) of batter protein was extractable in medium A (Fig. 1). A further 23% of could be extracted with aqueous ethanol (Fig. 1). A minor fraction (7%) was extracted in medium C (Fig. 1), and after reduction with DTT (medium D) the remaining fraction (16%) was extracted (Fig. 1). The obtained extraction yields corresponded well to the sum of the proteins extracted from the separate ingredients.

Extractability of Protein from Cake. After baking, little protein was extractable in media A, B, and C (5, 4, and 7% respectively; Fig. 1). About 80% of the protein in cake could be extracted after reduction of the SS bonds with medium D (Fig. 1). During baking, many batter proteins thus form covalent bonds that render them unextractable when only breaking up hydrogen and ionic bonds and hydrophobic interactions (using media A, B, and C). These results are in line with earlier observations (Deleu et al. 2015) that hardly any protein is extractable from pound cake under nonreducing

Fig. 1. Protein extraction yield, calculated for each medium as the ratio of extracted to total protein in egg white, egg yolk, wheat flour, and freeze-dried and defatted batter and cake after subsequent extraction with (A) 0.40M sodium chloride in sodium phosphate buffer (0.10M, pH 7.6) (SPB), (B) aqueous ethanol (60% v/v), (C) 2.0% (w/v) sodium dodecyl sulfate in SPB, and (D) 2.0% (w/v) dithiothreitol in SPB. Extraction yield from egg white protein with media B, C, and D were not determined (N.D.).

Fig. 2. Protein extraction yield, calculated for each medium as the ratio of extracted proteins to total protein in the initial sample, from freeze-dried and defatted cake after subsequent extraction with (C) 2.0% (w/v) sodium dodecyl sulfate (SDS) in sodium phosphate buffer (0.10M, pH 7.6) (SPB), (D) 2.0% (w/v) dithiothreitol (DTT) in SPB, or (E) 2.0% (w/v) SDS and 2.0% (w/v) DTT in SPB. Different (sequential) combinations of media were used: C and E, D and E, C and D, and only E.

conditions. The thermal denaturation was shown to play an important role in these changes (Deleu et al. 2015).

Based on these results, one can hypothesize that hydrophobic interactions (broken with SDS in medium C) play only a limited role in the cake structural network. To further investigate this, cake protein extractability was studied by using different combinations of SDS and DTT. When cake protein was extracted during the first extraction step in medium C or medium D (Fig. 2), only low protein yields were obtained (14 and 9%, respectively). However, Figure 2 shows that the remaining protein could be extracted in medium E, containing both SDS (present in medium C) and DTT (present in medium D). All protein was extracted under reducing conditions (medium D) following prior extraction with a medium containing SDS (medium C) (Fig. 2). So, breakage of SS bonds only resulted in a high extraction yield in the presence of SDS. When medium E was used on cake as the first extraction medium, all protein was extracted (Fig. 2). Because all protein was extractable using both (first or simultaneously) SDS and (then) DTT, the protein network in cake is most probably primarily based on SS bonds and hydrophobic interactions. However, hydrogen bonds and ionic bonds may also play a role.

Use of ^{15}N -Labeling to Distinguish Between Proteins from Different Ingredients. *^{15}N -Enrichment of Egg White and Egg Yolk.* During the period of labeled feeding (18 days), the enrichment of both egg white and egg yolk increased (Fig. 3). This was in line with literature data (Gruhn and Richter 1977; Fromentin et al. 2012). Egg white was highly enriched in ^{15}N after two days of ^{15}N -leucine-enriched feeding (Fig. 3). Egg white protein is found in the laid egg within two days after synthesis (Gruhn and Richter 1977; Sauveur 1988). Owing to transamination in the hen, the ^{15}N -label (Gruhn and Hennig 1984) redistributes and results in the egg proteins being uniformly labeled (Evenepoel et al. 1998).

In contrast to what was the case for egg white, the ^{15}N -enrichment of the egg yolk was much slower (Fig. 3). Egg yolk is built up of different oval layers, which are assembled during 8–10 consecutive days (Sauveur 1988). This way, enrichment of egg yolk in the secreted eggs increases rather linearly. Because the daily layers have the same protein composition, we do not expect differences in enrichment of the different yolk proteins. This was supported by the outcome of the analysis of separated plasma and granules (results not shown), for which the enrichment was comparable.

After 10 days of labeled feeding, both egg white and yolk were highly enriched (APEs 1.24 and 0.99, respectively; Fig. 3). Upon further labeled feeding, the enrichment still steadily increased to 1.37 and 1.19 for egg white and egg yolk, respectively. The enriched egg white and yolk after 10 days of feeding were separated and used in pound cake making.

Composition of the Batter Protein Extracts. The batter protein extractable in medium A originated from egg white (50%), yolk (32%), and flour (18%) (Fig. 4). When batters contained either labeled egg white or egg yolk, only negligible levels of ^{15}N were detected in the protein extracts in medium B. The fraction B con-

tains almost exclusively (98%) flour protein, more specifically gliadin (Fig. 4). This extract is further considered to be pure wheat protein, mainly gliadin. The protein extracted in medium C originated from the three ingredients, of which egg yolk (35%) and flour (54%) were the most abundant (Fig. 4). However, the total level of batter protein in this fraction was low (7%; Fig. 1). Fraction D contained mainly flour protein, more specifically glutenin (Fig. 4). Hardly any egg white or yolk proteins were found in this fraction because they were already extracted with the other media (Fig. 5).

Protein Distribution in Pound Cake and Its Batter. In pound cake batter, most (94%) egg white protein in cake batter was extracted in medium A (Fig. 5). The remainder was present in fractions C and D (3 and 2%, respectively; Fig. 5) and was most probably ovomucin and its different subunits. This is in line with the results of Figure 1.

A majority (83%) of the yolk protein in batter was extracted in medium A, whereas media C and D extracted 11 and 5%, respectively (Fig. 5). The protein level in fraction C was thus higher in batter

Fig. 4. Composition of the different extracts after subsequent extraction of batter protein with (A) 0.40M sodium chloride in sodium phosphate buffer (0.10M, pH 7.6) (SPB), (B) aqueous ethanol (60% v/v), (C) 2.0% (w/v) sodium dodecyl sulfate in SPB, and (D) 2.0% (w/v) dithiothreitol in SPB. This was determined by comparing the ^{15}N -enrichment of the extracts with that of the labeled egg protein used in the batter. Combining the data for egg white, egg yolk, and the total extractability, the extractability of the unlabeled wheat flour protein was calculated as the difference between total batter and egg protein.

Fig. 5. Relative extractability of egg white, egg yolk, and flour proteins from freeze-dried and defatted batter with (A) 0.40M sodium chloride in sodium phosphate buffer (0.10M, pH 7.6) (SPB), (B) aqueous ethanol (60% v/v), (C) 2.0% (w/v) sodium dodecyl sulfate in SPB, and (D) 2.0% (w/v) dithiothreitol in SPB. This was determined by comparing the ^{15}N -enrichment of the extracts with that of the labeled egg protein used in the batter. Combining the data for egg white, yolk, and the total extractability, the extractability of the unlabeled wheat flour protein was calculated as the difference between total batter and egg protein.

Fig. 3. ^{15}N -enrichment of egg white and egg yolk, expressed as atom percentage excess (APE), as function of the days of labeled feeding.

(11%; Fig. 5) than in yolk (4%; Fig. 1) extracts, whereas the level in fraction A was lower. Small differences in yolk protein induced by batter preparation were detected by using the ^{15}N -labeled protein from fresh eggs. The margarine in the batter may have led to (partial) protein denaturation (Dauphas et al. 2006). Hesso et al. (2015) already showed that much of the protein is affected by the fat in pound cake batter. Batter preparation may thus have induced not only changes in the egg yolk protein structure but also in its extractability.

The extractability of the (unlabeled) flour protein from the batter was calculated as the difference between the total batter protein and the egg proteins. Flour protein (Fig. 5) was mainly extracted in its Osborne fractions (Fig. 1).

^{15}N -labeling (Fig. 5) confirmed that the distribution of proteins from the different ingredients in the batter extracts was in essence that of the separate ingredients (Fig. 1). These data support the belief that protein network formation (by SS crosslinking) is very limited during batter preparation (Wilderjans et al. 2013; Hesso et al. 2015). In batter, wheat albumin and globulin, egg white protein, and most yolk protein are most likely dissolved in the aqueous phase, where it does not irreversibly react with other components at room temperature. Nevertheless, if present in the same phase, it is likely that the proteins interact and/or react with each other during baking. However, it is unclear whether or not the egg yolk granules disintegrate in batter and whether granule proteins are thus suspended as aggregates or rather dissolved in the aqueous phase.

As a result of baking, the protein extractabilities in the different media changed tremendously (Fig. 6 versus Fig. 5). The extractability of egg white protein in medium A decreased from 94% to only 4% (Fig. 6). This decrease coincided with an increase in extractability under reducing conditions (from 2 to 94% in medium D; Fig. 6). Thus, as a result of baking, 92% of the egg white protein was incorporated into the protein network by forming SS bonds. All egg white proteins have intramolecular SS bonds, but ovalbumin is the only one that has free SH groups. Therefore, it can form intermolecular SS bonds both by SH oxidation and SH/SS exchange reactions. The other egg white proteins can be incorporated into the protein network through SH/SS exchange reactions. Previous research showed that (S-)ovalbumin plays a key role in network formation. The heat-induced reactivity of ovalbumin is crucial for the overall network formation during cake baking, and it has been hypothesized that exposure of SH groups by ovalbumin is key (Deleu et al. 2015).

After baking, 77% of the egg yolk protein was only extractable under reducing conditions (medium D; Fig. 6). These proteins are

thus linked by SS bonds. Yolk protein is found in almost equal quantities in plasma or granules (Anton 2013). This implies that many proteins of both plasma and granules are incorporated by hydrophobic interactions and SS bonds. However, native granule proteins are organized in aggregates held together with ionic phosphocalcic bridges (Huopalahti et al. 2007). The question here is whether or not granules are disintegrated during cake making, and if so, whether they are able to react with proteins from other ingredients or only with other granule proteins. Some yolk proteins remained extractable in media A and C (7 and 15%, respectively; Fig. 6) after baking. These probably include some of high-density and/or low-density lipoproteins, but mainly phosvitin. The latter can be extracted after granule disintegration in media of high ionic strength (Huopalahti et al. 2007). Phosvitin makes up about 11% of the egg yolk protein and contains no SH groups or intramolecular SS bonds (Huopalahti et al. 2007). This makes it impossible to take part in the formation of intermolecular SS bonds.

There was a lower level of extractable wheat albumin and globulin (medium A, 7% in cake instead of 20% in batter) and gliadin (medium B, 8% instead of 44%) after baking (Fig. 6). The majority (77%) of flour proteins were only extractable under reducing conditions (medium D, Fig. 6). The extractable gliadin fraction made up about 18% of the initial gliadin population. Reversed-phase HPLC data obtained as in Wieser et al. (1998) showed that this fraction corresponded to the ω -gliadin, 16% of the gliadin population in the used flour (results not shown). ω -Gliadin has no cysteine and can therefore not participate in SS bonds. The other gliadins (α - and γ -gliadins), also extracted from batter in medium B, did form new interactions and/or reacted with other proteins during baking, including the formation of SS bonds.

CONCLUSIONS

The use of ^{15}N -labeled egg proteins allowed distinguishing between the sources of the proteins in batter and cake. This showed that hardly any changes in extractability were induced by the batter-mixing process. Protein network formation (by SS crosslinking) is therefore considered to be very limited during batter preparation.

During baking, a protein network is formed based on hydrophobic interactions and SS bonds. Almost all egg white protein is incorporated into the SS network, as deduced from its changed extractability in different media. Both egg yolk plasma and granule proteins are bound by SS bonds during baking. However, it remains unclear whether granules are disintegrated and this way can react with proteins from other ingredients. Much of the wheat flour albumin, globulin, and gliadin are incorporated into a network during baking.

Few proteins remain extractable when breaking hydrogen and ionic bonds and hydrophobic interactions. These are most probably phosvitin and ω -gliadin (i.e., proteins that have neither SH groups nor intramolecular SS bonds and thus lack the potential to form intermolecular SS bonds).

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Fig. 6. Relative extractability of egg white, egg yolk, and flour proteins from freeze-dried and defatted cake with (A) 0.40M sodium chloride in sodium phosphate buffer (0.10M, pH 7.6) (SPB), (B) aqueous ethanol (60% v/v), (C) 2.0% (w/v) sodium dodecyl sulfate in SPB, and (D) 2.0% (w/v) dithiothreitol in SPB. This was determined by comparing the ^{15}N -enrichment of the extracts with that of the labeled egg protein used in the batter. Combining the data for egg white, yolk, and the total extractability, the extractability of the unlabeled wheat flour protein was calculated as the difference between total batter and egg protein.

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