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Integrating the information from proteomic approaches: A "thiolomics" approach to assess the role of thiols in protein-based networks

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ABSTRACT

Thiol–disulfide exchange reactions, are major contributors to the formation of a covalently-linked protein network in many foods, where disulfides represent the most "natural" type of interprotein covalent bond. Thiol–disulfide exchange reactions occur also as a function of the accessibility of the involved thiols, which in turn depends on structural features of the involved proteins. Thiols in soluble and insoluble food proteins were covalently labeled by 5-iodoacetamide-fluorescein in the absence or in the presence of 4 M urea, a procedure that allowed to evaluate thiols accessibility before and after protein unfolding and dissociation of non-covalently linked protein complexes. Proteins labeled under either condition, along with unlabeled proteins, were then solubilized by treatment with disulfide reductants (and urea, when not added before) and separated either by SDS-PAGE or by two-dimensional electrophoresis. The 5-iodoacetamidofluorescein labeling procedures were also applied to soft wheat flours, and to semolina from durum wheat. Results highlight the different accessibilities of thiols in specific protein components in these materials, suggesting a possible role of minor protein components as for promoting rearrangement in the thiol pattern in wheat proteins upon processing and pointing out the relevance of structural issues in addition to compositional ones.

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1. Introduction

Cysteine thiols and cysteine disulfides represent the most "natural" way for generating covalently-linked protein networks in the most diverse foods. Thiol–disulfide exchange events are involved in a range of process-dependent molecular transformations in systems that range from whey proteins to egg proteins, and include water insoluble proteins such as those present in cereals. The network-forming capacity of proteins involved in thiol–disulfide exchange reactions in individual food systems is related to a multiplicity of factors, that include their relative abundance, the amount (and location) of reactive thiols and disulfides, and their availability to exchange events. Some of these parameters may be sensitive to process-induced structural modifications of the involved proteins, that may lead to exposure of reactive thiols or to their burial inside the structure of individual proteins and of protein aggregates (Iametti, Cairoli, De Gregori, & Bonomi, 1995; Iametti, De Gregori, Vecchio, & Bonomi, 1996). Structural modifications leading to exposure/burial of potentially reactive thiols have been addressed as a function of physical and chemical denaturation in a rather ample collection of soluble food proteins (Iametti et al., 1996, 1999). However, systems comprising water insoluble proteins (such as those in many cereals) are much more difficult to address, although their investigation is of paramount practical and economical significance.

The unique properties of proteins in wheat (and in related cereals) are instrumental to the production of extremely diverse common foods. The ability to form a viscoelastic network called gluten among gliadins and glutelins (Belton, 1999; Gobaa, Bancel, Branlard, Kleijer, & Stamp, 2008; Shewry, Tatham, Forde, Kreis, & Niflin, 1986) is quintessential to the consumer appreciation of the final product, be that due to retention of gas bubbles in bread and baked products or to entrapment of swollen starch in pasta (Singh & MacRitchie, 2001). From a molecular standpoint, the interactions leading to the formation of the visco-elastic network of gluten involve rearrangement of hydrophobic contacts among proteins (or within individual proteins) and rearrangement of intra- and intermolecular disulfides and thiols in a disulfide exchange process that requires protein flexibility (provided by the addition of water) and the action of shear forces that act as "mechanical denaturants" during mixing (Morel, Redl, & Guilbert, 2002). Gliadins are characterized by having mostly intramolecular disulfides, whereas glutelins form large aggregates linked by intermolecular disulfides (Shewry, Halford, Belton, & Tatham, 2002).

Given the relevance of disulfides and thiols in these processes, chemical and biochemical oxidants and reductants have often been used as "ameliorants" of dough rheology (Lagrain, Brijs, & Delcour, 2006). Oxidants (such as bromate/iodate or hydrogen peroxide, that may also be produced "in situ by appropriate enzymes (Hanft &

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Köhler, 2006) promote the formation of disulfides, whereas reducing agents (such as ascorbate, cysteine, and free or protein-bound glutathione) (Grosch & Wieser, 1999; Li, Tsiami, Bollecker, & Schofield, 2004) increase the number of thiols and facilitate thiol–disulfide exchange reaction if added in catalytic amounts.

In common practice, the amount of proteins in wheat flour or semolina and the glutelin/gliadin ratio in wheat flour are considered key parameters to predict their best possible use (Veraverbeke & Delcour, 2002). However, these parameters are often elusive (Goesaert et al., 2005) and predictions made only on these basis have been proven untenable in many cases, also in consideration of environmental and agronomic parameters, that may affect protein expression or protein structure after proteins are made and deposited in the seed (Iametti et al., 2006; Zörb, Grover, Steinfurth, & Muhling, 2010). A number of proteomics-based studies have contributed to provide a comprehensive and detailed view of the protein patterns in wheat, again in an attempt to find possible correlations with the processing performance of flours (Dupont, Vensel, Tanaka, Hurkman, & Altenbach, 2011; Mamone, De Caro, Di Luccia, Addeo, & Ferranti, 2009; Zörb et al., 2010) but not always such a correlation was evident.

Among the many other parameters that need to be considered in this frame are those of greatest interest to the food biochemist, that relate to structural issues. These include the folllowing: 1) the role of non covalent (mostly hydrophobic) interactions in network formation (Bonomi, Iametti, Pagani, & Ragg, 2007; Bonomi, Mora, Pagani, & Iametti, 2004); 2) the availability of thiols and disulfide to exchange reactions, as determined by overall and local protein flexibility — in turn related itself to protein solvability and to the intensity of physical deformation (Kieffer, Schurer, Köhler, & Wieser, 2007); and 3) the possible role of low molecular weight thiols and of low-abundance thiol-rich proteins in facilitating thiol–disulfide exchange reactions (Gao et al., 2009).

Methods capable of evaluating protein surface hydrophobicity in systems made up of water-insoluble proteins have been developed, and have been applied to protein characterization in cereal-based starting materials (Bonomi et al., 2004, 2007; Iametti et al., 2006) and products (Mariotti, Iametti, Cappa, Rasmussen, & Lucisano, 2011) without resorting to protein separation that necessarily uses solvent systems that affect protein structure and interactions. Protein solubility in the absence/presence of chaotropes and in the absence/presence of disulfide reductants has been used to study hydrophobic interactions among proteins (Mariotti et al., 2011) and their impact on the rheological properties of dough (Bonomi et al., 2012). Also, accessibility of thiols to common colorimetric or fluorescent reagents has been studied as a function of added chaotropes and of reaction time to assess overall and local rigidity of proteins in either the starting material or the finished products (Bonomi et al., 2012; Iametti et al., 1996).

The present work combines some of these approaches, in an attempt to gather an improved vision of molecular features that contribute to the properties of these systems. In particular, visual inspection and quantitative comparison of the maps for proteins and for accessible thiols in different and variously treated starting materials were carried out to identify specific proteins or protein classes whose thiol functions may specifically contribute to differentiate among materials and to elucidate the molecular basis of some of their specific traits.

2. Materials and methods

2.1. Materials

Unless otherwise indicated, samples of durum wheat semolina or of soft wheat flour were obtained from local sources and are representative of material commonly used for pasta or baked making, as appropriate. The durum wheat semolina sample had a gluten quality of 88, evaluated by the Gluten Index direct method (ICC 158, 1995) carried out by using a Glutomatic System (Perten, Sweden) (ICC 158, 1995). The soft flour sample had alveographic parameters P/L = 0.55 and W = 260 detected according to AACC 58-30A (2004). Bovine betalactoglobulin was purified from whole unheated milk according to published procedures (Barbiroli et al., 2011).

2.2. Covalent attachment of the fluorescent thiol probe

Unless otherwise stated, buffer was 0.05 M sodium phosphate, 0.1 M NaCl, pH 7.2. In experiments aimed at fluorescent labeling of freely accessible thiols in flour and semolina, the sample (50 mg) was suspended in 0.9 mL of buffer. After adding 0.1 mL of 5 mM 5-iodoacetamidofluorescein (IAF, in dimethylformamide) the suspension was stirred for 120 min at room temperature, and untreated IAF was blocked by the addition of excess dithiothreitol (DTT, 0.02 mL, 0.25 M in buffer). One mL of 8 M urea in buffer was added to the resulting mixture (final urea concentration, 4 M), and stirring was continued for 120 min at room temperature. In experiments aimed at labeling both freely accessible cysteine thiols and cysteine thiols made accessible only after breaking hydrophobic interactions among proteins or between different regions of the same protein (see Fig. 1), the sample (50 mg) was suspended in 0.9 mL of buffer containing 4 M urea. Following addition of 0.1 mL of 5 mM IAF, the suspension was left under stirring for 120 min at room temperature and excess IAF was blocked by the addition of 0.02 mL of 0.25 M DTT in buffer. Stirring was continued for another 120 min after diluting the suspension with an equal volume of 4 M urea in buffer.

A fluorescein-labeled derivative of bovine betalactoglobulin (BLG, containing a single free thiol on Cys 121 (Brownlow et al., 1999) was prepared by treating the protein (1 mg/mL or 0.045 mM, in buffer containing 4 M urea) with 0.5 mM IAF for 30 min at 60 °C. After blocking excess IAF with 5 mM DTT, the fluorescent BLG (containing 1 mol fluorescein/mol protein) was used as a fluorescence intensity standard in SDS-PAGE.

2.3. SDS-PAGE and 2DE

Materials from each of the labeling procedures described above were used for SDS-PAGE and two-dimensional electrophoresis (2-DE). Samples for SDS-PAGE were prepared by diluting solutions from the IAF treatment (after removal of insoluble materials by centrifugation at 5000 ×g for 10 min) with an equal volume of denaturing buffer (0.125 M Tris–HCl, pH 6.8, 50% glycerol (w/v), 1.7% SDS (w/v), 0.01% Bromophenol Blue (w/v)), followed by treatment at 100 °C for 5 min. Electrophoretic runs were performed at pH 8.3 (0.025 M Tris–HCl, 0.192 M glycine, 0.1% (w/v) SDS), in a Miniprotein II cell (Bio-Rad), at a constant 16 mA.

In the case of 2-DE, a 0.03 mL aliquot of solutions from either IAF treatment was mixed with 0.095 mL of rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS (w/v), 65 mM DTT, 2% IPG buffer pH 3–10, and 0.002% Bromophenol Blue (w/v). The sample was then loaded on 7 cm IPG strips (GE Healthcare) with a linear 3–10 pH gradient. Focusing was carried out in an Ettan IPGphor II apparatus (GE Healthcare). Prior to the second dimension, strips were incubated in equilibration buffer (0.375 M Tris–HCl, pH 8.8, 6 M urea, 2% SDS, and 20% glycerol) containing 65 mM DTT for 15 min. Then, strips were incubated with the equilibration buffer containing 0.243 M iodoacetamide for 10 min. Self-cast neutral pH gels were used for second dimension separation runs according to Holtzhauer (Holtzhauer, 2006).

Fluorescence patterns of SDS-PAGE tracings and 2DE gels were acquired by using a VersaDoc™ Image Analysis System (Bio-Rad), and analyzed through the Quantity One 1-D Analysis Software (BioRad, for SDS-PAGE) or the 2D ImageMaster Platinum (GE Healthcare). Gels analyzed for fluorescent spots were subsequently stained with a modified colloidal Coomassie Brilliant Blue according to published

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