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Proceeding Paper

Shotgun Proteomics: A Powerful Tool for Investigating the Chemical Complexity of Biscuit Melanoidins †

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Abstract: Melanoidins are the final products of the Maillard reaction (MR), a non-enzymatic browning reaction that occurs during food thermal processing, including biscuits, which are widely consumed. This work developed a novel technique for extracting biscuit melanoidins. Using shotgun proteomics, proteins involved in melanoidin formation were studied, and potential MR-induced protein modifications were investigated to elucidate the chemical structure and formation of biscuit melanoidins. It was observed that gluten proteins and soluble wheat flour-derived proteins were involved in melanoidin formation, along with the detection of protein modifications. These findings highlight shotgun proteomics as a promising tool for understanding biscuit melanoidins.

Keywords: Maillard reaction; melanoidins; biscuits; gluten proteins; protein modifications; shotgun proteomics

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1. Introduction

The Maillard reaction (MR) occurs during the thermal processing of various foods, initiated by the interaction between the carbonyl group of reducing sugars and the amine group of amino acids and proteins. Melanoidins are the end-products of this reaction and are defined as high molecular weight (HMW), brown-colored nitrogen-containing molecules [1]. Biscuits are widely consumed, especially among younger populations, making it crucial to study the chemical structure and formation mechanism of biscuit melanoidins for a better understanding of their impact on children's health. This work aims to develop a method for extracting melanoidins from biscuits and investigate the proteins involved in their formation and any modifications that may occur during MR. The shotgun proteomics technique will be used to characterize a protein mixture by analyzing peptides released through proteolysis using high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS).

2. Materials and Methods

2.1. Sample Preparation

Three biscuits were baked using commercial wheat flour following the official AACC method [2] at 205 °C for different durations (Biscuit A, BA—10 min; Biscuit B, BB—15 min and Biscuit C, BC—20 min). The biscuits were ground, defatted with n-hexane, and freeze-dried. Both the biscuit samples and the wheat flour used in baking were subjected to further fractionations.

2.2. Sample Fractionation

Sample fractionation comprised four steps, as summarized in Figure 1. After each step, precipitates and supernatants were dialyzed and freeze-dried. Extract yields were calculated based on the percentage of flour used in baking and the total material obtained in each extraction.

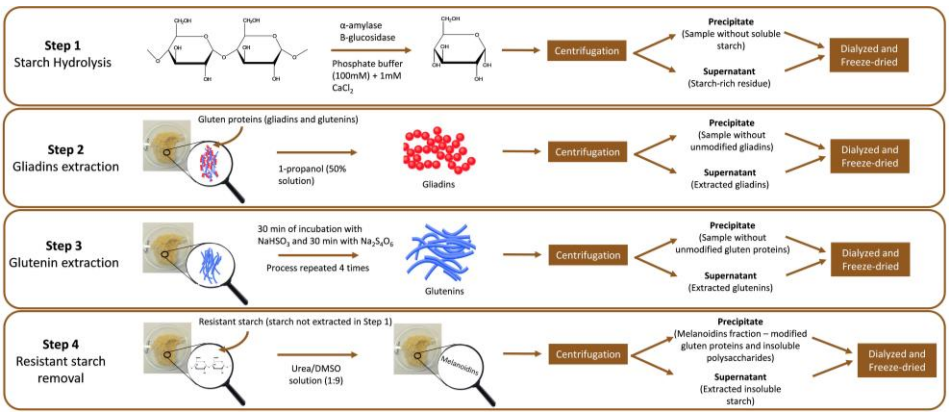

Figure 1. Summary of the four fractionation steps to which biscuit and flour samples were subjected.

2.3. Color Measurement

The color of the freeze-dried extracts from each fractionation step was evaluated using a spectrometer STD Vis/Nir with an optical fiber probe (Sarspec, Vila Nova de Gaia, Portugal) to measure the CIEL*a*b* parameters. The Browning Index (BI) was then determined based on these parameters to identify brown color changes among the fractions [3].

2.4. Peptide Analysis

The precipitates from the first and last step (initial fraction and melanoidin fraction) of each sample were incubated with trypsin, and the resulting peptides were analyzed by shotgun proteomics [4].

2.5. Identification of Proteins and Possible Modifications Induced by MR

Two software tools, Search Gui and Peptide Shaker, were used to identify proteins within the fractions, using the OMSSA searching algorithm [5] to find potential matches in a database containing *Triticum aestivum* from SWISS-PROT, as well as gliadins and glutenins from TrEMBL. The matches were validated with a 95% confidence level, and a 1.0% false discovery rate was applied to the peptide spectrum matching (PSM), peptides, and proteins. To assess the method's effectiveness in detecting protein modifications within the fractions, model systems were created. These systems involved reacting solutions of bovine serum albumin (BSA) and gluten with various compounds, including glucose, fructose, glyoxal, methylglyoxal, furfural, and glycolaldehyde. Tryptic peptides resulting from these reactions underwent the same analytical method as described earlier. Subsequently, the software tools were used to investigate specific modifications known to be induced by these compounds.

3. Results and Discussion

3.1. Fractionation Yields

It is hypothesized that melanoidins in wheat-based products form through crosslinking with gluten proteins [1]. Sample fractionation was based on the classical extraction of gluten proteins rather than common pronase E hydrolysis [1]. This approach enables the search for modifications in these proteins and enhances the understanding of the formation mechanism of melanoidins in these foods. It was observed that the extraction of gliadins decreases linearly with long baking time (Figure 2a). The extraction of glutenins remained constant among the different samples, except in the case of biscuit B, where a decrease

was observed. The melanoidin fraction increases with a longer baking time, rising by 66% during the first baking time (BA) compared to the flour (in the case of flour, the "melanoidin fraction" actually consists of insoluble polysaccharides, such as cellulose from the cell wall, accounting for 2% of the original sample). Figure 2b reveals that the most predominant extract from the flour was gliadins and glutenins, followed by non-extractable material. In the case of biscuits, the non-extractable material is the predominant fraction. This non-extractable material in biscuits has a strong brown color, higher than any fraction and even higher than the original biscuit, showing that is composed of a newly formed material and brown. Therefore, this fraction represents the biscuit melanoidins.

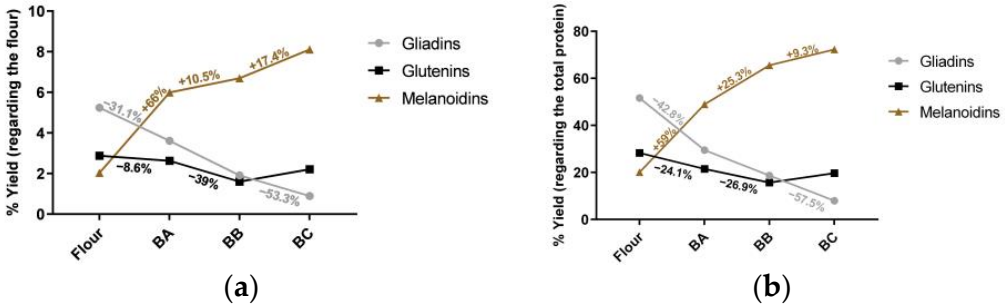

Figure 2. (**a**) Fractionation yields of each sample, based on the flour used in baking; (**b**) Fractionation yields relative to the total material obtained in each extraction step (Gliadins + Glutenins + Melanoidin fraction).

3.2. Color of the Samples

BI increases with longer baking times. There is a noticeable increase in browning between the initial fraction and the melanoidin fraction, as expected since melanoidins are more concentrated in the latter extract. Furthermore, an increase in browning is observed in both the gliadin and glutenin fractions. This phenomenon can be attributed to the fact that certain modified proteins can maintain their solubility, allowing them to remain extractable during the extraction process [6].

3.3. Identification of the Proteins of the Fractions

Using the shotgun proteomics method, it was possible to identify a large quantity of gluten proteins, mainly gliadins, in both the initial fraction (Figure 3b), and the melanoidin fraction (Figure 3c). This confirms their role in the formation of biscuit melanoidins.

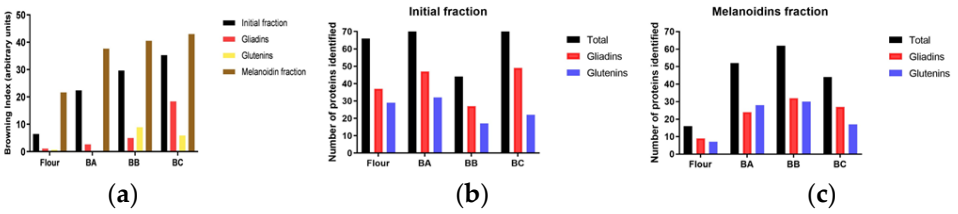

Figure 3. (**a**) Browning index obtained for each fraction of each sample; (**b**) Number of proteins identified in the initial fraction of each sample; and (**c**) Number of proteins observed in the melanoidins fraction of each sample.

3.4. Assessment of Protein Modifications Induced by MR

Some protein modifications, which have already been described in the literature as induced by the compounds used in the model systems, were found in the BSA and gluten reactions. For instance, a peptide modified with carboxymethyllysine (CML) was observed in the BSA + Glyoxal model system (Figure 4a), fructosyllysine (FL) modification in the BSA + Glucose model (Figure 4b), and pyrraline (Pyr) modification in an HMW glutenin, from the Gluten + Fructose model (Figure 4c). These findings show the suitability of this method for identifying protein modifications induced by MR in biscuit samples. An example of

this is the detection of a peptide from a gamma-gliadin containing ArgPyrimidine, in the melanoidin fraction of BB (Figure 4d).

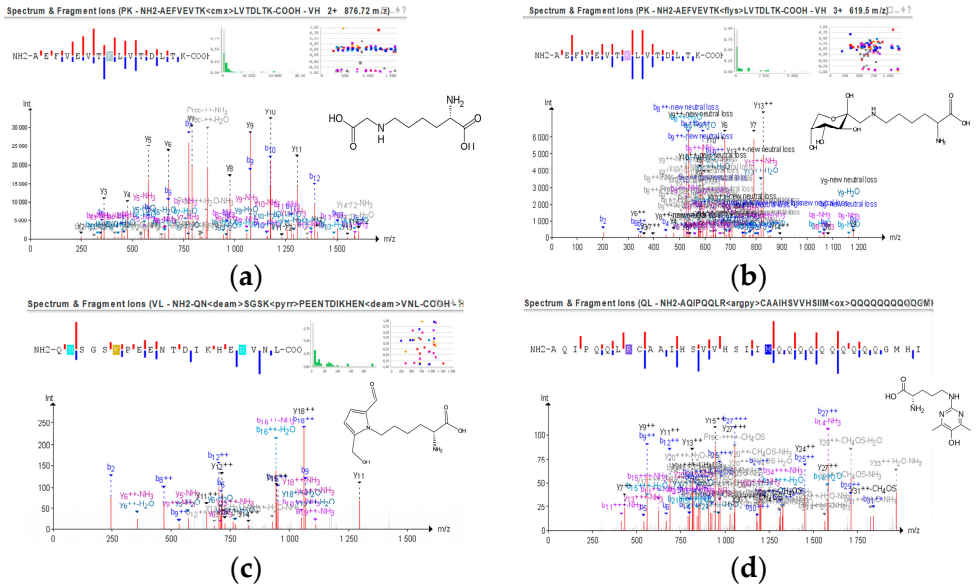

Figure 4. (**a**) Peptide from the BSA + Glyoxal model with CML; (**b**) Peptide from the BSA + Glucose model with FL; (**c**) Peptide of an HMW glutenin, from the Gluten + Fructose model, with Pyr; and (**d**) Peptide from a gamma-gliadin in the melanoidin fraction of BB, with ArgPyrimidine.

4. Conclusions

In this work, it was observed that the melanoidin content of the biscuits increased with baking time, resulting in samples browning, while the extracted gliadins decreased. A variety of gluten proteins were identified in the biscuits, indicating their incorporation into biscuits melanoidins. The shotgun proteomics method demonstrated promise for identifying proteins in melanoidin fractions and has been successfully validated for searching protein modifications induced by the MR in a simple and non-destructive way.

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