Fluorescence Fingerprint Imaging of Gluten and Starch in Wheat Flour Dough with Consideration of Total Constituent Ratio

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An imaging method using fluorescence fingerprints (FFs) for visualizing the distribution of gluten and starch in dough without any staining was developed. Wheat flour dough was mixed up to three stages, i.e., under-mixing, optimum-mixing, and over-mixing, and thin sections of the doughs were prepared with a cryotome. Fluorescence images of the sections were acquired in 63 combinations of excitation and emission wavelengths, thereby constructing the FFs of the constituents at each pixel. The cosine similarity between the FFs of each pixel and the FF of pure gluten were calculated and pixels were arranged in order of cosine similarity. Pixels with higher values of cosine similarity were categorized as “gluten” and the rest as “nongluten”. The number of pixels categorized as “gluten” was based on the overall ratio of gluten in the dough. The same process was performed with the FF of pure starch, and all pixels were divided into “starch” and “nonstarch”. Colors were assigned to each division, and the distributions of gluten and starch were visualized. Changes in the distributions of gluten and starch were observed at the over-mixing stage, which suggested the breaking up of gluten and the alteration of gluten and starch.

Keywords: excitation-emission matrix, imaging, autofluorescence, cosine similarity

Introduction

Bread palatability is determined by many factors, one of which is bread texture (Babin et al., 2005). Bread texture largely depends on the condition of the dough from which bread is made, particularly the rheological properties of the dough. In wheat flour dough, the microscopic spatial arrangement of gluten and starch greatly affects dough rheology, and many advanced microscopic techniques have been applied to observe the microstructure of dough (Peighambardoust et al., 2010).

Specific chemical compounds in the dough are mainly visualized by combining light microscopy and staining. Many approaches to light microscopy have been used to study dough, such as bright field (Auger et al., 2008; Kuktaite et al., 2005), confocal scanning laser (Durrenberger et al., 2001; Peressini et al., 2008; Renzetti and Arendt, 2009) and epifluorescence light (Peighambardoust et al., 2010) microcopies. Stains are used to differentiate between constituents of interest such as gluten and starch; however, they may introduce sample alteration or artifacts. The results could also vary depending on the selected stains and staining conditions such as concentration, solvent and staining time.

Specific constituents have been visualized without staining by hyperspectral or multispectral imaging, a method that integrates conventional imaging with spectroscopy (Gowen et al., 2007). Hyperspectral or multispectral images are composed of multiple wavebands for each spatial position studied, and light absorption/fluorescence/reflectance at these wavebands give intrinsic information on the constituent at that position.

Recently, fluorescence fingerprints (FFs) have been used as spectroscopic data in hyperspectral imaging to visualize internal structures (Tsuta et al., 2007) and specific constitu-
ents (Kokawa et al., 2011). FF, also known as the excitation-emission matrix (EEM), is a set of fluorescence spectra acquired at consecutive excitation wavelengths, creating a three-dimensional diagram (Jiang et al., 2010). The pattern of this diagram is unique for every constituent, and several FF studies of various foods have been reported (Sadecka and Tothova, 2007) such as meat (Moller et al., 2003), olive oil (Guimet et al., 2004), and buckwheat noodles (Shibata et al., 2011).

FF has been used in imaging to visualize the internal structure of soybean seeds (Tsuta et al., 2007) and the distribution of gluten and starch in dough (Kokawa et al., 2011; Kokawa et al., 2012). In these studies, the FF data acquired at each spatial position are analyzed to obtain information on the target constituent, and this information is visualized by converting the calculated values into pseudo color.

In this imaging process, the colors in the pseudo color image do not correspond to absolute quantities of the constituents, but only to relative quantities between each pixel, e.g. pixel 1 contains more of the constituent than pixel 2. To create an image that shows absolute quantities of the constituents at each pixel, it would be necessary to build a strict calibration model that links spectral data with constituent quantity. However, building a calibration model requires the preparation of several samples with different quantities of the target constituent (Kokawa et al., 2013), which could be difficult with real samples. Thus, in this study, we aimed to develop an imaging method that does not require complex calibration and could be applied to real samples, but still reflects the absolute quantities of the constituents in the sample.

**Materials and Methods**

**Sample preparation** Three batches of dough at the under-, optimum-, and over-mixing stages were prepared from flour (Camellia, Nisshin Flour Milling Inc., Tokyo, Japan) and tap water, as reported earlier (Kokawa et al., 2011). The dough was cut into small pieces, approximately 2 cm square, frozen quickly at −80°C to minimize the change in dough microstructure through freezing, and kept at the same temperature.

Gluten and primary starch were fractionated from dough as explained by Kokawa et al. (2011). Gluten was cut and frozen as described above. The primary starch was freeze-dried and kept at −20°C. These samples of fractionated gluten and starch were used as reference data when analyzing the FF of the dough.

Thin sections were made from the dough, gluten and starch samples with a cryomicrotome (Leica CM1850, Leica Microsystems Japan, Tokyo, Japan) according to Kokawa et al. (2011). Thin sections were needed because it was shown in a previous study (Kokawa et al., 2013) that starch and gluten on the surface of the sample could not be observed with fluorescence when samples were thick. This was because the penetration depth of excitation light was larger than starch granules and gluten strands, and picked up fluorescence from all the constituents underneath.

**Measurement of gluten to starch ratio in dough** Three batches of dough were made from 50.00 g of flour (Camellia) and 32.50 g of distilled water. The dough was soaked in distilled water for 2 h to strengthen the connection of gluten. After soaking, the dough was carefully kneaded in water to separate gluten from starch granules. The retrieved gluten was removed of excess water on the surface and then weighed. The water with the precipitated starch granules were poured into eight centrifuge tubes (50 mL) for each batch of dough and centrifuged for 10 min at 7000 rpm. The water was drained for 30 min and the precipitated starch was weighed. Both gluten and starch were weighed in a wet state.

**Acquisition of fluorescence images** An FF imaging system consisting mainly of a near-ultraviolet light source (MAX-302, Asahi Spectra, Tokyo, Japan), a monochrome CCD camera (ORCA-ER-1394, Hamamatsu Photonics, Shizuoka, Japan), and two sets of band-pass filters was constructed as reported earlier (Kokawa et al., 2011; Kokawa et al., 2012). With this imaging system, light from the light source is filtered through a band-pass filter (HQBP filter, Asahi Spectra) and light of a specific wavelength is irradiated onto the sample. The fluorescence emitted from the sample is filtered through another band-pass filter (M.C. Filter, Asahi Spectra) to obtain a fluorescence image of the sample at a specific emission wavelength. By sequentially changing the band-pass filters on the excitation and emission sides, multiple images of the sample at different excitation and emission wavelengths can be acquired. When the measurement process is complete, the set of fluorescence images acquired at all combinations of excitation and emission wavelengths in the FF can be viewed as a set of FFs for every pixel.

The excitation and emission wavelengths used for fluorescence image acquisition were 260 to 320 nm and 370 to 450 nm, respectively, at 10 nm intervals. These seven excitation wavelengths and nine emission wavelengths were combined to make 63 wavelength conditions (combinations of excitation and emission wavelengths). The exposure time of the camera was set at 1.0, 5.0, or 10 sec, longer for wavelengths at which the fluorescence emission was weak and shorter for those at which fluorescence emission was strong. The exposure time for the same wavelength condition was uniform throughout all samples. The set of fluorescence images was taken using 2 × 2 binning (Nasibov et al., 2010), resulting in an image of 512 × 672 pixels. Dark noise (Sugiyama, 1999; Tsuta et al., 2002) was corrected by subtracting a “dark image” acquired by the same camera while the camera shutter...
was closed and the lens covered with aluminum foil.

Fluorescence images of thin sections of dough (three mixing stages), gluten and starch were acquired.

Image processing and calculation of cosine similarity  The fluorescence images were analyzed with versatile numerical analysis software (MATLAB, The MathWorks, Massachusetts, USA), and the analytical process followed the process explained earlier (Kokawa et al., 2011).

First, the positions of the FF images were adjusted to correct for the slight shift of the measuring device (objective lens, CCD camera, etc.), which occurred each time the fluorescence filter was changed. The correction was made using the image registration tools in MATLAB. A region of 256 × 336 pixels was selected from the center of the original image for further analysis.

Next, bubble areas were masked using linear discriminant analysis. Since the bubble areas were relatively dark in all the fluorescence images, discriminant analysis was based on the Euclidean norm of FFs. To perform discriminant analysis, a training set was made by selecting areas that were clearly inside the bubble or sample areas. From the FFs of the pixels, a discriminant function was calculated, which was then used to classify border pixels between the bubble and sample areas. The bubble areas were excluded from the following analysis.

Finally, the values of cosine similarity (Cao et al., 2008; Li et al., 2007) between the FFs of each pixel in the dough image and those of gluten and starch were calculated. The average FFs of gluten and starch were used in the calculations.

Results and Discussion

Ratio of gluten to starch in dough  The average ratios of gluten to starch were 20.52% and 79.48%, respectively. The reproducibility between the three batches of dough was good, the standard deviation being 0.35%. These values agree with the literature values (Macritchie, 1985).

The water contents of the retrieved gluten and starch were 64.8% and 50.7%, respectively. The standard deviations were 0.64% and 1.0% for gluten (three samples) and starch (five samples), respectively. Since gluten and starch absorb different amounts of water (Kokawa et al., 2013), the ratio of gluten to starch would be different between wet and dry states. In this study, the visualized dough sample existed in a wet state. Therefore, the retrieved gluten and starch were measured without force-drying the samples.

Visualization of distributions of gluten and starch  The value of cosine similarity between the FF of a pixel in the dough sample and the FF of gluten (or starch) can be interpreted as the possibility of gluten (or starch) actually existing in the area. This means that the larger the value of cosine similarity, the higher the possibility of existence.

Figure 1 shows the cosine similarity image of dough in the under-mixing stage, and the histogram of cosine similar-

Fig. 1. Cosine similarity images of dough in the under-mixing stage, and histograms of cosine similarity values. The red and green images show cosine similarity values to gluten and starch, respectively. By considering the gluten to starch ratio of the dough, pixels were divided into “gluten” or “nongluten”, and “starch” and “nonstarch”.

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ity values. Considering the ratio of gluten to starch obtained from the fractionation experiment (20.52% to 79.48%), 20.52% of pixels with a higher value of cosine similarity to gluten were labeled as “gluten” and the rest, “nongluten”. Similarly, 79.48% of pixels with a higher value of cosine similarity to starch were labeled as “starch” and the rest, “nonstarch”. The “gluten” and “nongluten” pixels were colored in red and dark red, respectively. The “starch” and “nonstarch” pixels were colored in light and dark green, respectively.

For each pixel, there are four possible combinations, as can be seen in Figure 2: “nongluten” and “nonstarch”, “nongluten” and “starch”, “gluten” and “nonstarch”, and “gluten” and “starch”. The colors chosen for each combination are shown in Figure 2. These were determined by assigning channels R and G of the RGB color coordinate system to gluten and starch, respectively, and setting “gluten” or “starch” to 255, and “nongluten” or “nonstarch” to 64 (0.25*255). The value of 0 was saved for bubble areas. Channel B was set to 0.

Figure 3 shows images of the dough in the under-, optimum-, and over-mixing stages. The orange areas show a high similarity to gluten and a low similarity to starch, suggesting the existence of gluten. The green areas show an opposite tendency, suggesting that starch exists at that point. The yellow areas show a high similarity to both gluten and starch, probably showing that both constituents exist in nearly equal amounts. The dark green areas that show low similarities to both gluten and starch also suggest a mixture of gluten and starch. These areas may also be other constituents that show a relatively strong but different fluorescence pattern from gluten and starch, such as particles of the aleurone layer (Irving et al., 1989).

Since the resolution of the images is limited, and features that are smaller than a pixel cannot be visualized, thin strands of gluten, which surround starch granules (Amend and Bellitz, 1990) cannot be observed in the images. However, the magnification and resolution of the images can be altered by changing the magnification of the objective lens and binning parameters of the CCD camera, and more detailed images may be acquired in future studies.

**Quantification of changes in dough through mixing** The gluten particles (orange) that can be seen in the under-mixing and optimum-mixing stages almost disappear in the over-mixing stage. In contrast, the yellow areas increase significantly. These observations were confirmed by calculating the number of pixels falling in each category for each mixing stage.

Figure 4 shows the number of pixels in each category throughout the three mixing stages. There is a significant change in the number of pixels falling in each category from the optimum-mixing stage to the over-mixing stage: the orange and green pixels decrease, while the yellow and dark-

![Fig. 2. Combinations of “gluten” or “nongluten”, and “starch” or “nonstarch”, and the resulting colors. Each pixel was assigned a category based on the cosine similarity to gluten and starch, and colored accordingly.](image1)

![Fig. 3. Images of dough in the (a) under-, (b) optimum-, and (c) over-mixing stages.](image2)
green pixels increase in number. Bubble area also increases, which is in agreement with the result of a previous study (Kokawa et al., 2012).

The decrease in the numbers of orange and green pixels and the increase in the numbers of yellow and dark-green pixels suggest that the gluten breaks up into small pieces in the over-mixing stage and mixes with starch granules, so that in most of the pixels there is a mixture of gluten and starch. The breaking up of gluten with excessive mixing has been observed by SEM (Amend and Belitz, 1990).

The threshold values of cosine similarity that divide gluten from nongluten and starch from nonstarch changed markedly from the optimum-mixing stage to the over-mixing stage. The threshold values for gluten are 0.9915 and 0.9894 for the under- and optimum-mixing stages, respectively, but these values decrease to 0.8697 in the over-mixing stage. Similarly, the threshold values for starch are 0.9301 and 0.9261 for the first two mixing stages, respectively, but are 0.8016 in the over-mixing stage. This suggests that, in the over-mixing stage, gluten and starch themselves are altered from the gluten and starch fractionated from the optimally mixed dough.

**Conclusion**

In this study, a novel FF imaging method for visualizing gluten and starch in wheat dough was developed. The cosine similarities between the FF of each pixel in the dough and the FF of gluten (or starch) were calculated, based on the assumption that the larger the cosine similarity, the higher the possibility of gluten (or starch) existing at that spot. 20.52% of the pixels with the largest similarity to gluten were categorized as “gluten”, and the rest, “nongluten”; 70.48% of the pixels with the largest similarity to starch were categorized as “starch”, and the rest, “nonstarch”. These numbers were derived from the actual ratio of gluten to starch in the dough, obtained by fractionation. Each pixel was colored according to the assigned category, thereby visualizing the distributions of gluten and starch. By applying this imaging method to the analysis of dough in the under-, optimum-, and over-mixing stages, the breaking up of gluten and the alteration of both gluten and starch were shown to proceed from the optimum-mixing stage to the over-mixing stage. By assigning colors to pixels on the basis of the actual ratio of constituents of the whole sample, the ambiguity in the conversion of cosine similarity to color was resolved.

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