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Endosperm and aleurone cell structure in barley and wheat as studied by optical and Raman microscopy

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ABSTRACT

Grain ultrastructure is of utmost importance when designing grain processing procedures in the food industry. In this study, wheat and barley grain components were localised using optical and Raman microscopy. The optical microscopic analyses were performed using several selective stains to localise β-glucan, protein and starch or autofluorescence to image the ferulic acid and other fluorescing substances. Alternatively, Raman microscopy was applied to localise the grain components without any need for preceding staining or other sample pretreatment. Both methods provided consistent information on the grain structures, illustrating the distribution of polysaccharides, aromatics and protein in endosperm and aleurone layers. In aleurone layers of both barley and wheat, a distinct difference between the anticlinal and periclinal cell walls was observed. The anticlinal cell walls were enriched with aromatic substances which were present in remarkably lower concentrations in the inner periclinal cell walls but for barley, an even higher concentration in the outer periclinal cell wall was observed. In addition, Raman spectroscopy illustrated the detailed distribution of substances across the aleurone cell walls: β-glucan was adjacent to proteins and it was deficient in the middle lamella whereas arabinoxylan was enriched in the outer cell wall layers and middle lamella.

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1. Introduction

Wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) are among the most commonly cultivated agricultural cereal crops. Grain structure and composition determine hardness (Nair et al., 2011; Turnbull and Rahman, 2002) and hence the processability of the grains in industrial food processes such as in milling, breadmaking, malting and brewing. In addition, the grain composition affects the cereal digestion in human and animal gut and contributes to the nutritional value and possible health benefits.

Cereal grains contain starchy endosperm surrounded by the aleurone layer, seed coat and pericarp (Fincher and Stone, 1986). Endosperm comprises the major part of the cereal grain and it is composed of cells filled with starch granules embedded in a protein matrix. The endosperm cell walls consist mostly of arabinoxylan and β-glucan, while the content of other substances, such as phenolic acids, cellulose, heteromannans and proteins is very low (Fincher and Stone, 1986).

The aleurone layer contains cells in one, two or three rows for wheat and barley, respectively (Fincher and Stone, 1986). These cells are filled with proteins containing globoids which are composed mostly of phytin. Aleurone cell walls are composed mostly of arabinoxylan, β-glucan and phenolic acids which are mostly ferulic acid (90%) and coumaric acid (10%) while other phenolic acids, such as diferulic acids are observed only in trace amounts (Antoine et al., 2003; Hernanz et al., 2001). Aleurone cell walls also contain some proteins, cellulose and heteromannans (Fincher and Stone, 1986).

Maternal layers of grains (i.e. nucellar layer, seed coat, pericarp and hull) are composed of lignified cells (Fincher and Stone, 1986). Most of these cells are hollow and their cell walls are composed mostly of xylan (ca. 60%), cellulose (ca. 30%) and lignin (ca. 10%) (Fincher and Stone, 1986). Lignin is enriched in intercellular and primary wall layers, and it is present in lower concentrations in secondary walls. Phenolic acids are also concentrated in these outer layers of cereal grains (Nordkvist et al., 1984).

Optical microscopy is widely applied in characterizing cereal grain structures. Autofluorescence can be applied to illustrate the localisation of aromatic substances, such as phenolic acids and lignin. On the other hand, the localisation of other substances is most commonly performed by utilising selective stains which

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reveal the distribution of e.g. starch, polysaccharides or waxes. In a recent paper, Dornez et al. (2011a,b) compared different staining techniques for cereal crops. They illustrated that combination of several stains is a practical approach to localise multiple grain components simultaneously. For example, combining acid fuchsin and calcofluor illustrates the distribution of β-glucan and protein in the grains. However, no stains for certain cereal grain components exist. Localisation of arabinoxylan by optical microscopy is challenging since there are no specific dyes for its staining, and therefore its localisation requires immunolabeling or xylanase probe staining, which are more advanced and more tedious methods than the direct staining (Guillon et al., 2004).

Raman microscopy (Raman imaging or mapping) provides an alternative staining-free microscopic technique to image the distribution of different components in sub-micron resolution in different plant-based samples (Smith and Dent, 2005). In this technique, Raman spectra are collected at regular intervals of the sample and an image is reconstructed based on the spectral band intensities or other spectral features. Raman mapping has been applied to image several different types of plant sections (Gierlinger and Schwanninger, 2007) but only a few papers on Raman analysis of cereal grains exist. Wheat grains (Piot et al., 2000, 2002) and their isolated components (Barron and Rouau, 2008) have been studied by Raman spectroscopy, but no publications on cereal grain imaging with high spatial resolution (<1 μm) to reveal the cell wall ultrastructure exist. In addition, no Raman microscopic studies on barley grains have been published.

This paper illustrates the structural distribution of components in wheat and barley endosperm and aleurone cells by optical and Raman microscopy. Staining techniques were applied to identify the distribution of protein, starch and β-glucan in wheat and barley grains. This was followed by autofluorescent microscopic analysis to localise the aromatic and other fluorescing structures in the samples. Thereafter, Raman microscopic analysis was applied to the same grains to localise starch, proteins, ferulic acids, arabinoxylan and β-glucan simultaneously and to compare the results with the optical microscopic analysis.

2. Experimental

2.1. Grain sample preparation

Barley and wheat grains were fixed with 3.0% paraformaldehyde and 1.0% glutaraldehyde in 0.10 M sodium potassium phosphate buffer pH 7.0, dehydrated in a series of ethanol solutions and infiltrated with a Historesin Embedding Kit (Leica Microsystems, Mensheim, Germany) as described previously (Dornez et al., 2011a,b). The kernels were embedded in sample blocks and cut into thin sections (4 μm) with a rotary microtome HM355 (Microm Laboggeräte GmbH, Walldorf, Germany) using a steel knife. The sections were then transferred onto 2-welled microscopy slides and dried on a metal heating plate at 40 °C prior to staining or Raman imaging. 12 grains of each cereal were embedded and sectioned and imaged with optical microscopy with both staining methods.

2.2. Grain model substances

Raman spectral analyses were performed for the cereal grain model substances: gliadin (Sigma–Aldrich), hordein (Shewry et al., 1978), arabinoxylan from rye (*Secale cereale* L.) (Leuven), barley starch (Rajamäen), barley β-glucan (Megazyme), ferulic acid (Sigma–Aldrich) and phytic acid (Sigma–Aldrich). The rye-based ferulate-free arabinoxylan was selected as model substance in order to be able to study the changes in the degree of feruloylation within the samples.

2.3. Optical microscopy

Acid fuchsin – calcofluor staining was performed by applying 0.1% (w/v) acid fuchsin for 2.0 min followed by 0.01% (w/v) calcofluor white for 2.0 min to stain protein and β-glucan, respectively. After each staining, the sections were rinsed with deionised water and dried. The stained grain sections were imaged using an Olympus BX50 epifluorescence microscope (Olympus Corp. Tokyo, Japan). The samples stained with acid fuchsin and calcofluor were imaged using mirror cube U-MNV (Olympus; excitation 420–480 nm, emission $>$455 nm) and 100× magnification.

Light green – iodine staining was performed by staining protein with aqueous 0.1% (w/v) Light Green for 1 min (Gurr, BDH Ltd.) and starch with 1:10 diluted Lugol's iodine solution (I2, 0.33%, w/v; and KI, 0.67%, w/v). The samples were imaged with light microscope Olympus BX50 microscope (Olympus Corp. Tokyo, Japan) in brightfield illumination with 100× magnification (Holopainen et al., 2005).

Autofluorescence images were collected using an Olympus BX50 epifluorescence microscope (Olympus Corp. Tokyo, Japan) using 100× magnification, UV excitation at 330–380 nm and detecting the emission above 420 nm wavelength.

2.4. Raman spectroscopy and microscopy

Raman spectra of the model substance were collected using a Witec Alpha300 microspectroscope (Witec GmbH, Ulm, Germany) equipped with a 532 nm green laser and grating of 600 l/mm. The spectra were collected through a 20× air objective and the collection time was 20 s for a single spectrum. Several spectra were collected from different sample positions and the sum of these spectra was utilised in the analyses. No baseline correction or other spectral treatment was performed for the single spectra, except levelling the baseline to zero.

Raman spectral images were collected using the same spectroscope as above but using a 100× air objective, 0.5 s spectral collection time for each spectrum and 200 nm steps between each spectral collection point. The spectral images were 20 μm × 20 μm in size. Four to six parallel images were collected from each grain layer from at least two different grains.

Each spectrum in the spectral images was baseline corrected using 4th order polynomial fit to remove the background. The model spectra at a spectral range of 350–1900 cm^{-1} were utilised to calculate the Raman images using basis spectral analysis as has been described earlier (Österberg et al., 2006). In this procedure, each measured spectrum of the 2-dimensional data array is fitted by a linear combination of basis spectra S_k using the least squares method. Each compound has a unique Raman spectrum S_k, called a basis spectrum. A sample which consists of N different compounds shows a linear superposition of all its basis spectra $S = \Sigma a_k S_k$ where $k = 1$ to N depending on the number of compounds in the sample. If the basis spectrum S_k is known for each compound, it is possible to estimate the weight factor a_k by a least squares fit. The weighting factor is proportional to the quantity of the compound and is illustrated in the corresponding Raman image. Hence, the images were calculated using large spectral range of intense bands instead of single spectral bands of individual components which would partly overlap with signals from other components.

3. Results and discussion

3.1. Optical microscopy of wheat and barley grains

The optical microscopy images of barley and wheat grain cross sections are illustrated in Fig. 1. The uppermost images illustrate the

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