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Spectrophotometric and fluorimetric quantitation of quality-related protein fractions of wheat flour

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ABSTRACT

Spectrophotometric and fluorimetric methods for quantitating quality-related parameters of wheat flour such as the gliadin (GLIA), glutenin (GLUT), and glutenin macropolymer (GMP) contents were adapted. The bicinchoninic acid (BCA) assay, the Bradford assay, the fluorescamine assay, the Naphthol Blue Black (NBB) assay, and the acid orange 12 assay were studied. Calibration functions were generated by using protein references isolated from a mixture of flours of 10 wheat cultivars. Among the assays studied, only the Bradford, the fluorescamine and the NBB assays provided useful results. The Bradford assay succeeded in quantitating the GLIA and GLUT fractions, but it was unsuitable for the GMP fraction because of the presence of SDS. All relevant protein fractions could be quantitated with the fluorescamine and the NBB assays. While the former showed higher coefficients of variation, the NBB assay was more reliable. NBB and HPLC data as well as NBB and micro-scale baking test data were strongly correlated. The NBB assay is a good alternative to chromatographic methods, which require time and sophisticated equipment. It can be adapted as easy-to-use, high throughput method that enables screening of a large number of flour samples for quality parameters in a short time per sample.

1. Introduction

It is generally accepted that the baking quality of wheat flour is strongly correlated with the qualitative and quantitative composition of gluten proteins. More than 30 years ago Moonen et al. (1982) showed, that after extraction of wheat flour with a SDS-containing buffer and centrifugation, a gel-like layer, called gel protein or glutenin macropolymer (GMP), settled on the sediment starch. The concentration of this SDS-insoluble gel layer depended on the wheat cultivar, and a positive correlation (correlation coefficient: $r = 0.87$) of GMP amount to baking performance (bread volume) was established (Moonen et al., 1982). These results were supported by the work of MacRitchie and Lafiandra (1997), who demonstrated that the content of the high-molecular-weight part of the glutenin fraction, called unextractable polymeric protein (UPP), was highly correlated with dough resistance. Although isolation and nomenclature of UPP were somewhat different from those of GMP, these fractions represented more or less the same part of the glutenin fraction.

Previous investigations demonstrated that both gliadin (GLIA) and glutenin (GLUT) concentrations and proportions in wheat flour affect dough properties and bread volume (Wieser and Kieffer, 2001). In a recent study on 13 wheat cultivars, we have shown that GLIA and GLUT and additionally GMP concentrations in flour are strongly correlated with the baking performance. In particular GMP was found to be a good predictor of the baking performance of wheat flour (Thanhaeuser et al., 2014). In this work, GLIA and GLUT were isolated by a modified Osborne fractionation and quantitated by reversed-phase (RP-) HPLC according to Wieser et al. (1998). GMP was separated as residue after extracting flours with SDS-solution and quantitated by gel permeation (GP-) HPLC after reduction of disulfide bonds.

Laboratories of the milling industry are usually not equipped with RP- or GP-HPLC instrumentation, but spectrophotometers are available in almost every laboratory. Spectrophotometers could be used to determine GLIA, GLUT, and GMP contents as quality-related parameters of wheat flour, if suitable spectrophotometric methods for quantitation would be available. One approach using turbidimetry to quantitate GLIA and GLUT fractions in a spectrophotometer was reported by Wieser (2000). The main drawback of this method was that the precipitates required exact temperature control in the photometer to get reproducible readings. On the

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other hand, a considerable number of spectrophotometric methods for protein quantitation are routinely being used in protein biochemistry (Lowry et al., 1951; Bradford, 1976; Smith et al., 1985; Schaffner and Weissmann, 1973). However, adaptation of these methods to quantitate gluten protein fractions of wheat flour should include suitable protein references for calibration and should also take into account the poor solubility of gluten proteins in water or salt solutions and the requirement to use reducing agents or SDS to solubilize gluten proteins. Up to date, the use of spectrophotometric methods to quantitate GLIA, GLUT, and GMP in wheat flour has not been described.

Spectrophotometric and fluorimetric methods for protein quantitation include the bicinchoninic acid (BCA) assay (Smith et al., 1985; Sorensen and Brodbeck, 1986), the Bradford assay (Bradford, 1976), the fluorescamine assay (Udenfriend et al., 1972; Lorenzen and Kennedy, 1993), and the Naphthol Blue Black (NBB) assay (Schaffner and Weissmann, 1973). An assay using acid orange 12 (Udy, 1954) has been approved by the American Association of Cereal Chemists International (2000) as method no. 46-14.02 to determine the crude protein content of wheat flour. The structures of the dyes used in these methods are given in Fig. 1.

Rosell et al. (2013) were the first who described the use of the BCA assay to determine the content of wheat proteins in 1.5% SDS. In the BCA assay, Cu^{2+} ions are reduced by amino acid side chains of the proteins (cysteine, tryptophan, and tyrosine) yielding a violet complex between Cu^{+} and two BCA molecules, with a maximum absorbance at 562 nm. The Bradford assay uses Coomassie Brilliant Blue G-250, which binds to positively charged side chains of the proteins at low pH causing an absorption shift from 465 to 595 nm. Fluorescamine can be used for fluorimetric protein quantitation. This compound forms covalent bonds with primary amino groups of the proteins yielding a fluorescent pyrrolidone derivative within a very short time (Fig. 1). Different excitation/emission combinations can be used for quantitation: 390/475 nm (Udenfriend et al., 1972), 400/460 nm (Lorenzen and Kennedy, 1993), or 392/480 nm (Ferrari et al., 1998). Comparable to Coomassie Brilliant Blue the NBB dye interacts with positively charged amino acid side chains of the proteins at low pH. In contrast to the Bradford method, the NBB-protein complex precipitates. The complex is re-dissolved after isolation and washing by raising the pH-value. The amount of liberated NBB is proportional to the protein concentration and can be measured spectrophotometrically at 620 nm. Acid orange 12 is a protein specific dye forming an insoluble, ionic complex with basic amino acid side chains of the proteins. The amount of unbound dye (absorption at 480 nm) is inversely correlated to the amount of protein in the sample (Udy, 1954).

Altogether, the assays and dyes mentioned above react differently with proteins. Thus, the amino acid composition of a protein is an important property and determines the response of a dye with a given protein. Wieser et al. (1983) demonstrated that GLUT contains nearly twice the amount of positively charged side chains than GLIA. This means that proper calibration is necessary to get reliable protein concentrations. Thus, the respective protein fraction itself appears to be the best reference for calibration. Consequently, suitable reference proteins or protein fractions have to be prepared in sufficient amounts as part of the method development.

The aim of this study was to develop easy-to-use methods to evaluate protein-based, quality-related parameters of wheat flours based on spectrophotometry or fluorimetry. The parameters GLIA, GLUT, and GMP content should be determined. The novel methods should show at least an equal correlation with the baking performance than chromatographic methods used so far. Crucial points were the easy and quick feasibility and the availability of suitable reference proteins for proper calibration.

2. Materials and methods

2.1. Wheat samples

Grains from winter wheat cultivars officially approved in Germany (all baking performance classes; German Federal Plant Variety Office (2011)) were obtained from breeders. Thirteen cultivars harvested in 2010 (Tables 1 and 2) were used to adapt the spectrophotometric methods. Further, ten cultivars harvested in 2009 (*cultivars Akteur, Bussard, Cubus, Dekan, Herrmann, Manager, Mulan, Potential, Tommi, Winnetou*) were used to prepare reference proteins. Finally, a set of ten cultivars from the 2011 harvest (Table 5) was used to verify the spectrophotometric methods. Grains were milled into flour with a Quadrumat Junior Mill (Brabender, Duisburg, Germany). The flour was sieved (mesh size 0.2 mm) and allowed to rest for two weeks prior to use. The moisture, ash, and protein (N × 5.7) contents were determined

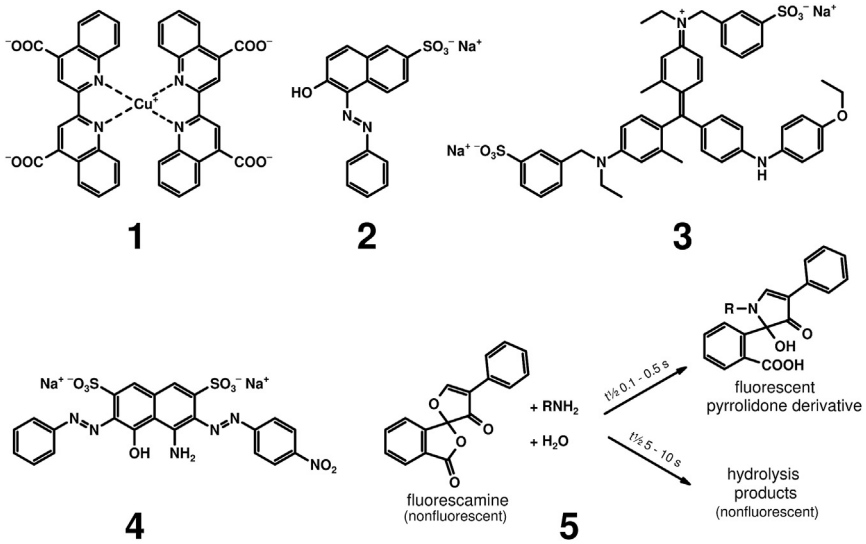

Fig. 1. Chemical structures of **1** bicinchoninic acid–copper complex, **2** acid orange 12, **3** Coomassie Brilliant Blue G-250, **4** Naphthol Blue Black 10B, and **5** the structure and reaction mechanism of fluorescamine.