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RESEARCH ARTICLE

Determination of soluble wheat protein fractions using the Bradford assay

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Abstract

Background and objectives: Determination of different grain protein fractions in wheat cultivars is an important task in analyzing bread baking quality. In many laboratories, the Bradford assay is used to determine protein concentrations in solutions. In any protein assay (including Bradford), the ideal protein to use as a standard is the purified protein being assayed. In the absence of such an absolute reference, protein another protein must be selected as a relative standard such as bovine serum albumin (BSA) which is widely used. The aim of this work was to find conversion factors for BSA to determine correct albumin–globulin, gliadin, and glutenin concentrations, because these purified wheat grain protein fractions are mostly not available to be used for calibration purposes.

Findings: In case of BSA calibration, gluten concentration was underestimated (50%–54%) compared to calibration with the respective purified wheat proteins (65%–70%) in extracts of wheat grain samples. This result is explained with the different amino acid composition of BSA and wheat protein fractions leading to a more intense signal with BSA in the Bradford assay. Calibration of the Bradford assay using BSA as well as purified wheat protein fractions allowed to calculate the conversion factors of 2.11 for BSA/albumin–globulin, 4.25 for BSA/gliadin, and 3.42 for BSA/glutenin. Application of these conversion factors proved to accurately adjust protein concentrations of wheat fractions originating from ten cultivars, determined with BSA calibration of the Bradford assay.

Conclusions: BSA calibration of the Bradford assay in combination with the conversion factors can be used to determine protein concentration of wheat grain fractions.

Significance and novelty: Findings of this study make a contribution toward the correction of a common method, to provide a basis for better comparability of research results from different laboratories.

KEYWORDS

albumin–globulin, Bradford assay, BSA, gliadin, glutenin, Osborne fractions

1 | INTRODUCTION

Determination of protein concentration of individual protein fractions of wheat grain is a very common task in wheat science, for example, for estimating baking potential and quality of wheat cultivars and species grown in various environments (Geisslitz et al., 2019; Langenkämper & Zörb, 2019). Often, reversed-phase high-performance liquid chromatography (RP-HPLC) and sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) methods are used to analyze the Osborne protein fractions of wheat grain: albumins-globulins (ALB-GLOB), gliadins (GLIA), and glutenins (GLUT) (Geisslitz et al., 2019; Jang et al., 2017; Pronin et al., 2020; Rekowski et al., 2019). While these methods reveal detailed information about the Osborne fractions, including concentrations of ALB-GLOB, GLIA, GLUT, and their respective subtypes, they require time-consuming as well as special and expensive laboratory equipment. Frequently, the total concentration of the different fractions and the ratio of GLIA/ GLUT is of interest. Directed at this goal, the simple-to-perform and cheap Bradford protein assay has been adapted to determine GLIA and GLUT concentrations in different wheat species (Geisslitz et al., 2019; Thanhaeuser et al., 2015). The authors calibrated the Bradford assay using isolated GLIA and GLUT fractions from common wheat. Subsequently, the Bradford assay and RP-HPLC analyses were performed in parallel on sets of samples from common wheat, as well as spelt, durum wheat, emmer, and einkorn. For both, GLIA and GLUT, high correlation coefficients (ranging from 0.779 to 0.944) were found across all species, when results of Bradford assays and RP-HPLC were compared. It was concluded that the Bradford assay based on calibration with the respective isolated protein fractions could be used reliably to determine concentrations of GLIA and GLUT in diverse wheat species (Geisslitz et al., 2019; Thanhaeuser et al., 2015).

The Bradford assay is a protein determination method that involves binding of the Coomassie Blue dye to proteins (Bradford, 1976), where the efficiency of dye binding depends on the amino acid composition of the protein. Coomassie Blue dye binds primarily to (a) basic amino acid residues (especially arginine, lysine, and histidine), (b) the terminal amino group in the polypeptide chain, and (c) aromatic amino acid residues (Compton & Jones, 1985). Quantification of protein in samples of interest with the Bradford assay requires calibration with a protein of known concentration. Since dye binding depends on the composition of amino acids, the ideal calibrant protein is a purified preparation of the protein being assayed, *for example,* isolated GLIA and GLUT fractions. However, such purified protein fractions are often commercially not available, and for a large number of laboratories, it is estimated to exceed their capabilities to produce sufficiently purified calibrant proteins in an appropriate amount. Bovine serum albumin (BSA) is probably the most common calibrant protein used in the Bradford assay and it has the advantage of being readily available at reasonable costs. One major drawback is that BSA and wheat proteins possess different amino acid compositions (Thanhaeuser et al., 2015), leading very probably to erroneous results, when determining wheat protein concentration purely based on BSA calibration.

The main objective of this work was to investigate whether BSA calibration of the Bradford assay could be used to accurately determine protein concentrations of the Osborne fractions of wheat. To achieve this goal, calibrations of the Bradford assay using BSA and isolated ALB-GLOB, GLIA as well as GLUT were constructed, with the aim to find constant conversion factors between these different calibrations. The established conversion factors were then tested on protein determination in a set of wheat samples originating from ten different cultivars.

It is known that certain chemical–dye interactions (such as some detergents, flavonoids, and basic protein buffers) interfere with the Bradford assay. Interference is due to the ability of these substances to shift the equilibrium levels by direct binding of the dye or by shifting the pH. Nevertheless, many chemical reagents do not directly affect the development of dye color when used at sufficiently low concentrations. Therefore, it is required to use the same composition of the different solutions of this work here, in order to be able to generally apply the conversion factors.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Bovine serum albumin (BSA) and purified wheat fractions (ABL-GLOB, GLIA and GLUT) were used for standard calibration curves. BSA was purchased from Sigma-Aldrich (BSA lyophilized powder fraction V, ≥98%, Sigma-Aldrich, Steinheim, Germany) while purified ALB-GLOB, GLIA, and GLUT were prepared from white flour of the cultivar Akteur with ALB-GLOB 57.8%, GLIA 91.0%, and GLUT 80.9% protein content, based on the study by Schalk et al., (2017).

The Roti-Quant reagent (five times concentrate) from Roth (Carl Roth, Karlsruhe, Germany) was used for the Bradford assay.

Different solvents were prepared for each individual protein fraction, where choice of solvent corresponded to the extraction procedure for the fractions according to Wieser et al. (1998): Solvent A (0.067 mol/L Na_2HPO_4/KH_2PO_4, 0.4 mol/L NaCl, pH 7.6) for ALB-GLOB, solvent B (70%

(v/v) ethanol) for GLIA, and solvent C (2 mol/L urea, 1% (w/v) dithiothreitol, 50% (v/v) 2-propanol, 0.05 mol/L Tris pH 7.5) for GLUT.

2.2 | Protein sample preparation for standard curves

Powders of BSA and the three wheat protein fractions were dissolved in the appropriate solvent, with the aid of an ultrasonic-bath incubation (640 watts, frequency KHz35, 10 min) (SONOREX SUPER RK 156, Bandelin electronic GmbH & Co. KG, Berlin, Germany) to yield protein stock solutions. The BSA stock solution (10 mg/ml in H_20 of double distilled) was serially diluted in H_2O (double distilled) to yield concentrations of 1; 0.75; 0.5; 0.25 and 0.125 µg/µl. ALB-GLOB stock solution was prepared in solvent A at 2.5 µg/µl (accounting for the protein content of 57.8% of the ALB-GLOB standard), followed by a serial dilution in solvent A to 2.0; 1.5; 1.0 and 0.5 µg/µl. Similarly, GLIA stock solution was made up to 5 µg/µl in solvent B (accounting for the protein content of 91.0% of the GLIA standard) and then serially diluted in solvent B to 3.9; 2.8; 1.7 and 0.6 µg/µl. Finally, GLUT stock solution in solvent C contained 5.0 µg/µl (accounting for the protein content of 80.9% of the GLUT standard), and dilutions in solution C were done at 4.0; 3.0; 2.0 and 1.0 µg/µl, respectively. Bradford assays (see Chapter 2.3) were performed for each dilution series in order to obtain stand curves.

2.3 | Sample preparation for Bradford assays and photometric measurements

The Bradford assay is supplied as a 5x concentrate. In our work here, assays were performed in a total volume of 1,000 µl (semi micro cuvette), containing 200 µl of Bradford reagent. The following pipetting sequence was applied: H_2O (770 µl); solvent A, B, or C (20 µl); protein sample in solvent A, B, or C (in 10 µl); vortex, Bradford reagent (200 µl), vortex, incubation for 11 min, measurement in the photometer (Specord® 50 Plus, Analytik Jena, Jena, Germany) at $\lambda = 595$ nm. In the Bradford assay, comparative photometric measurements of blank values with the three solvents (A, B, or C) against blank values with water have shown that the solvent volumes must not be neglected. The maximum solvent volume in this study did not exceed 30 µl, and this volume was obeyed for blanks and samples containing protein. Protein sample amount could be adjusted within the set volume of 30 µl in order to reach a photometric reading within the range of the corresponding calibration curve. Each sample was assayed in four replications. If standard deviations of the four replications exceeded 5%, the assay for the sample was repeated. For all blank value measurements, first the photometer was set to zero at $\lambda = 595$ nm against air. Then, the reading of the blank value should be in the range of 0.80 ± 0.10. If this was the case, the photometer reading was set to zero.

2.4 | Extraction of protein fractions from wheat samples and determination of protein concentration

Wheat grains (*Triticum aestivum*. L), originating from ten different cultivars and different experimental set up, were chosen to examine, whether the BSA calibrated Bradford assay could reliably be used to determine protein concentration in wheat protein fractions. Grains were milled with a ball mill (MM301, Retsch, Haan, Germany) into wholemeal flour. In order to achieve the best possible homogenization for subsequent protein extraction, the grinding process was carried out in two steps with a total duration of 90 s with a frequency of 27 Hz and a pause of approximately 30 s to prevent denaturation of the proteins caused by heating of the samples during grinding.

The three protein fractions ALB-GLOB, GLIA, and GLUT were extracted, according to Osborne, (1907) based on a modified procedure from Wieser et al. (1998). For the extraction of ALB-GLOB, 1 ml of extraction buffer (0.067 mol/L Na_2HPO_4/KH_2PO_4, 0.4 mol/L NaCl, pH 7.6) was added to 200 mg of the wholemeal flour, mixed in an overhead shaker (Multi Bio RS-24, bioSan, Riga, Latvia) for 5 min at 20°C, and incubated on ice for 10 min with repeated vigorous vortexing. After centrifugation (13,800 g, 6°C, 10 min), the supernatant containing ALB-GLOB was transferred into new tubes, and the entire extraction step was repeated two more times.

The remaining pellet was then extracted with 0.8 ml of 70% (v/v) ethanol for 5 min at 20°C in an overhead shaker with repeated vigorous vortexing and centrifuged again at 13,800 g (6°C, 10 min) to yield the GLIA fraction. Again, the extraction of the sediment was repeated two more times. After a washing step with 1 ml of double distilled H_2O and centrifugation (13,800 g, 6°C, 5 min), the GLUT fraction was extracted using 0.8 ml of extraction buffer (2 mol/L urea, 1% (w/v) dithiothreitol, 50% (v/v) 2-propanol, 0.05 mol/L Tris pH 7.5) for 5 min in an overhead shaker at 20°C, followed by incubation at 60°C for 10 min, cooling to room temperature (RT), and centrifugation at 13,800 g (6°C, 10 min). As before, the extraction of the sediment was done three times. The supernatants of each of the three extraction steps were kept separately. Finally, all samples were frozen at −20°C for later use. Two separate extractions (*i.e.*, technical replicates) were done for each sample.

The Bradford assay was used to determine the concentration of protein fractions spectrophotometrically ($\lambda = 595$ nm). The

concentration of all samples was calculated on basis of the BSA as a calibrant and on the basis of the purified wheat protein fractions ALB-GLOB, GLIA and GLUT as calibrants. All samples were measured in four replications to reduce technical errors.

The nitrogen content of the flours was determined according to Kjeldahl, using a VAPODEST® 50s (C. Gerhardt GmbH & Co, Königswinter, Germany). The nitrogen content was multiplied by the factor 5.7 to obtain the crude protein content.

3 | RESULTS AND DISCUSSION

3.1 | Standard curves and calculation of conversion factors

Standard curves were obtained for BSA as well as for purified ALB-GLOB, GLIA, and GLUT as calibrants applying the Bradford assay. The BSA standard curve showed the steepest slope (m = 0.0616) compared to standard curves for the wheat protein fractions, which were decreasing in the order ALB-GLOB (m = 0.0292), GLUT (m = 0.0180), and GLIA (m = 0.0145, (Figure 1). Further, all four calibration curves had an y-intercept very close to zero (Figure 1), rendering the effect of the y-intercept negligible, when comparing the slopes. Thus, in dividing the BSA slope by each individual slope, the conversion factor for the specific protein fraction is obtained. Division of the BSA slope by the ALB-GLOB slope resulted in 2.11 (BSA/ALB-GLOB factor). The same was done for GLIA (0.0616/0.0145 = 4.25, BSA/GLIA factor) and GLUT (0.0616/0.0180 = 3.42, BSA/GLUT factor) as well. It is obvious that BSA calibration would underestimate (due to the steeper slope) the concentration of all three wheat protein fractions, if not corrected with an appropriate conversion factor, respectively. This can be explained with the difference in the amino acid composition of the wheat protein fractions and BSA. An "in silico" comparison of arginine, histidine, and lysine contents of BSA (accession P02769: Arg 3.9%, His 2.9% Lys 10.1%) with some representative GLIA (alpha/beta-gliadin, accession P18573: Arg 1.7%, His 1.4% Lys 0.3% and gamma-gliadin, accession P21292: Arg 1.4%, His 2.5% Lys 0.7%) and GLUT (high molecular weight glutenin, accession P10388: Arg 1.2%, His 0.5% Lys 0.7% and low molecular weight glutenin, accession Q8W3V2: Arg 2.8%, His 1.1% Lys 0.4%) illustrates the significantly lower content of basic amino acids of the wheat grain proteins.

3.2 | Concentration of individual protein fractions based on different standard curves and application of the conversion factors

The following experiments were performed in order to test whether the calculated conversion factors could be validated, when applied to wheat grain samples from ten different wheat cultivars. The protein fractions were extracted from wholemeal flour, and subsequently, the protein concentrations of the different fractions of all cultivars were determined using the slope and y-intercept derived from the four equations (Figure 1). For all fractions, protein concentrations were either calculated with BSA as a calibrant (Table 1.a) or with the respective protein as a calibrant (Table 1.b). If BSA was used as a calibrant, expectedly, the sum of the concentrations of the three different fractions in flour was lower than the crude protein content determined with the Kjeldahl method in all ten cultivars (with the ratio of approximately 3.3). In contrast, when the respective protein was used as a calibrant the sum of ALB-GLOB, GLIA, and GLUT matched the crude protein content almost one-to-one (Table 1.b). Considering the percentage of gluten in both, Table 1a and 1b, it is revealed that in case of BSA calibration, gluten concentration is underestimated (50%–54%) compared to calibration with the respective

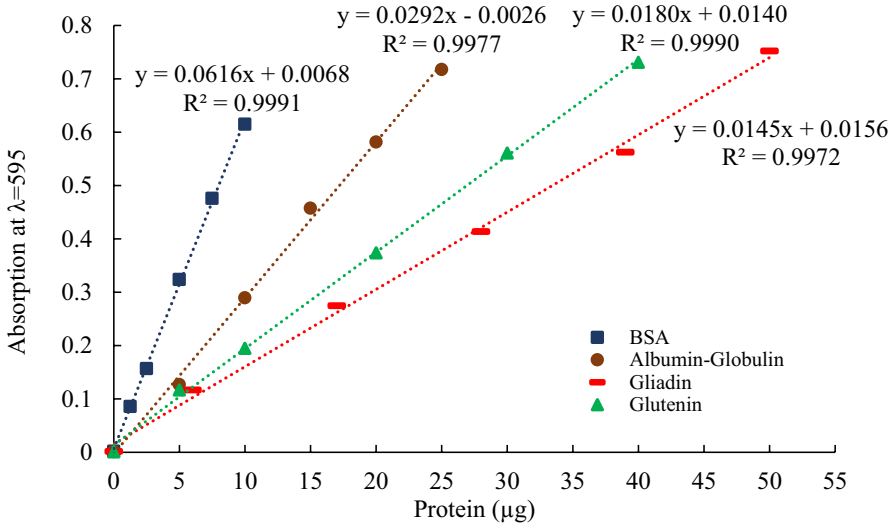

FIGURE 1 Linear graphs obtained with the Bradford assay using BSA (blue) and the purified wheat protein fractions Albumin–Globulin (brown), Gliadin (red) and Glutenin (green) as calibrants. Slope and y-intercept from the four derived equations. All values were measured in four replicates

TABLE 1 Crude protein concentration and concentrations of individual protein fractions for the ten different wheat cultivars based upon the Bradford assay. (a) using BSA as a calibrant and (b) using the respective purified wheat proteins (ALB-GLOB, GLIA, or GLUT) as calibrants. All values were measured in four replicates

(a)

No	Cultivar	Crude Protein (Kjeldahl)	ALB-GLOB	GLIA	GLUT	ALB-GLOB+GLIA+GLUT	ALB-GLOB+GLIA+GLUT	Ratio Kjeldahl to Bradford	[a]ALB-GLOB	[b]GLIA	[c]GLUT	[d]Gluten
		%	mg/g flour	mg/g flour	mg/g flour	mg/g flour	%		%	%	%	%
1	Alvand	15.0	21.9	11.4	11.7	45.1	4.5	3.3	48.7	25.3	26.0	51.3
2	Mihan	14.0	18.4	11.7	12.9	43.0	4.3	3.3	42.7	27.3	30.0	57.3
3	Akteur	14.1	21.7	11.5	10.8	44.0	4.4	3.2	49.3	26.2	24.5	50.7
4	Rumor	15.4	21.7	10.5	15.1	47.3	4.7	3.3	45.8	22.3	31.9	54.2
5	Patras	15.3	22.7	11.7	14.6	49.1	4.9	3.1	46.3	23.9	29.8	53.7
6	Anapolis	13.8	20.5	10.7	12.5	43.7	4.4	3.2	46.9	24.6	28.5	53.1
7	Discus	14.8	22.1	11.6	13.8	47.5	4.8	3.1	46.5	24.5	29.0	53.5
8	Impression	15.2	23.3	10.9	15.8	49.9	5.0	3.0	46.6	21.7	31.7	53.4
9	Hybrey	14.3	23.1	10.6	12.8	46.5	4.7	3.1	49.7	22.9	27.5	50.3
10	Hystar	15.2	23.2	11.5	14.9	49.5	5.0	3.1	46.8	23.2	30.0	53.2

(b)

No	Cultivar	Crude Protein (Kjeldahl)	ALB-GLOB	GLIA	GLUT	ALB-GLOB+GLIA+GLUT	ALB-GLOB+GLIA+GLUT	Ratio Kjeldahl to Bradford	[a]ALB-GLOB	[b]GLIA	[c]GLUT	[d]Gluten
		%	mg/g flour	mg/g flour	mg/g flour	mg/g flour	%		%	%	%	%
1	Alvand	15.0	45.6	48.3	40.1	134.0	13.4	1.1	34.0	36.0	29.9	66.0
2	Mihan	14.0	39.8	50.0	43.5	133.3	13.3	1.1	29.8	37.5	32.6	70.2
3	Akteur	14.1	45.5	50.5	37.2	133.2	13.3	1.1	34.2	37.9	27.9	65.8
4	Rumor	15.4	45.2	45.0	53.1	143.3	14.3	1.1	31.5	31.4	37.1	68.5
5	Patras	15.3	46.4	49.5	50.6	146.4	14.6	1.0	31.7	33.8	34.5	68.3
6	Anapolis	13.8	42.5	47.0	43.8	133.3	13.3	1.0	31.9	35.2	32.8	68.1
7	Discus	14.8	46.6	51.3	46.6	144.5	14.5	1.0	32.2	35.5	32.3	67.8
8	Impression	15.2	49.8	47.0	52.6	149.4	14.9	1.0	33.3	31.5	35.2	66.7
9	Hybrey	14.3	48.7	47.1	43.6	139.3	13.9	1.0	34.9	33.8	31.3	65.1
10	Hystar	15.2	49.1	48.8	47.8	145.7	14.6	1.0	33.7	33.5	32.8	66.3

[a]Albumin-Globulin proportion (%) = albumin-globulin (mg/g flour)/total grain protein (mg/g flour) × 100.

[b]Gliadin proportion (%) = gliadin (mg/g flour)/total grain protein (mg/g flour) × 100.

[c]Glutenin proportion (%) = glutenin (mg/g flour)/total grain protein (mg/g flour) × 100.

[d]Gluten proportion (%) = gliadin proportion (%) + glutenin proportion (%).

TABLE 2 Application of the three BSA/wheat protein conversion factors on protein determinations based on BSA calibration of the Bradford assay

No	Cultivar	ALB-GLOB mg/g flour	Difference %	GLIA mg/g flour	Difference %	GLUT mg/g flour	Difference %
1	Alvand	46.2	1.3	48.5	0.3	40.0	0.2
2	Mihan	38.8	2.5	49.7	0.6	44.1	1.4
3	Akteur	45.8	0.6	48.9	3.2	36.9	0.7
4	Rumor	45.8	1.3	44.6	0.8	51.6	2.7
5	Patras	47.9	3.2	49.7	0.5	49.9	1.3
6	Anapolis	43.3	1.8	45.5	3.2	42.8	2.4
7	Discus	46.6	0.1	49.3	3.9	47.2	1.3
8	Impression	49.2	1.3	46.3	1.4	54.0	2.7
9	Hybrey	48.7	0.1	45.1	4.4	43.8	0.4
10	Hystar	49.0	0.3	48.9	0.2	51.0	6.6
Mean	-	46.1	1.2	47.6	1.8	46.1	2.0

Note: Originating from Table 1a, ALB-GLOB data were multiplied with the BSA/ALB-GLOB factor (2.11), GLIA data were multiplied with the BSA/GLIA factor (4.25), and GLUT data were multiplied with the BSA/GLUT factor (3.42). Differences (%) compared to data of Table 1b are shown.

purified protein (65%–70%). The gluten concentration ranges of 65%–70% reported in this study fall well within the gluten content range of 60%–80%, reported for wholemeal flour of different wheat cultivars (Geisslitz et al., 2019).

Finally, the protein concentrations of the different fractions of ten wheat cultivars obtained with BSA calibration (Table 1.a) were multiplied with the conversion factors for BSA/ALB-GLOB (2.11), BSA/GLIA (4.25), and BSA/GLUT (3.42), and the results are presented in Table 2. After application of the conversion factors, there was only a small difference compared to the protein determination data using the purified wheat proteins as calibrant (Table 2) The differences, averaged across all ten cultivars, were 1.2% for ALB-GLOB, 1.8% for GLIA, and 2.0% for GLUT (Table 2). Considering these relatively small variations, the conversion factors established within this work here can be applied in case of BSA calibration of the Bradford assay, to yield accurate protein concentration data for the wheat grain protein fractions ALB-GLOB, GLIA, and GLUT. Since the Bradford assay is sensitive concerning presence of solvents, salts, and detergents, it is required to use the same composition of the different solutions of this work here, in order to be able to generally apply the conversion factors.

4 | CONCLUSION

Researcher having no access to purified ALB-GBLO, GLIA, and GLUT proteins may use a BSA calibration of the Bradford assay in combination with the conversion factors established in this work here, in order to obtain more accurate results when determining concentrations of ALB-GBLO, GLIA, and GLUT in extracts of wheat grain. The availability of these conversion factors will provide a basis for better comparability of research results concerning wheat grain protein fractions from different laboratories.

It has to be mentioned that the purified wheat fractions (ABL-GLOB, GLIA, and GLUT) used in this study were prepared from white flour of the cultivar Akteur with European origin. It is advisable to follow-up the current work with a larger sample set of wheat from different origins (e.g., North America) or different quality classes to confirm the conversion factors.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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