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Technical Note

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Fast and Sensitive Total Protein and Peptide Assays for Proteomic Analysis

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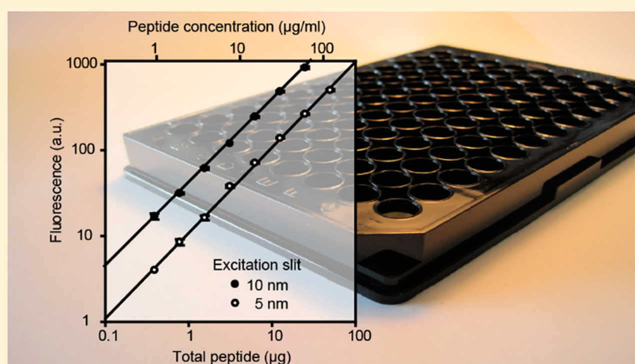
ABSTRACT: The determination of total protein content is one of the most frequent analytical tasks in biochemistry and molecular biology. Here we evaluate measurements of tryptophan fluorescence (WF) for total protein determination in whole tissue lysates and for peptide quantification in protein digests. We demonstrate that the fluorescence spectrometry of tryptophan offers a simple, sensitive, and direct method for protein and peptide assays. The WF assay is fully compatible with SDS and other solutes that are commonly used for lysis of tissue and cells. We found that the content of tryptophan varies only a little between mouse tissues (1.16 ± 0.08% of total amino acids) and is similar in human cells (1.19 ± 0.06%). Therefore, free tryptophan can be used as a universal standard. We show that the assay can be carried out on a standard fluorescence spectrometer with cuvettes as well as in a 96-well format using a plate reader. The method is particularly suitable for determination of peptide content in diluted samples. Notably, the whole sample can be recovered after the measurement.



Total protein determination belongs to the group of most frequently carried out assays in biochemistry and molecular biology. Because of its importance, numerous assays have been developed, and several of them are commonly used. Broadly, the protein assays can be divided into two categories: the UV absorbance methods and colorimetric or fluorimetric dye-binding assays. (For an overview, see ref 1.) Although determination of the total protein in body fluids and other solutions of proteins can be carried out directly by UV spectrometry, whole cell lysates of tissues cannot be assayed in this way. Indeed, the presence of nucleic acids, which strongly absorb light in the same range as proteins, interferes with their detection. In contrast, the dye-binding assays allow protein determination by measuring absorbance or fluorescence in the visible light region, thereby avoiding this interference.

The direct UV assay is less sensitive than the dye-based methods but allows almost complete recovery of the sample after the measurement. In contrast, this is not the case for the dye-based assays and becomes a critical point when samples containing only minute amounts of protein have to be assayed. In addition, the colorimetric assays often interfere with substances used for tissue lysis such as detergents or disulfide-bond-reducing agents. For example, the Bradford assay[2] is not compatible with SDS, whereas the bicinchoninic acid assay (BCA)[3] does not tolerate dithiothreitol (DTT), β-mercaptoethanol, or EDTA.[1]

Proteins contain three different aromatic amino acids carrying benzene, phenol, and indole rings, respectively. Each of these groups can be excited by UV light to fluoresce; however, mainly the fluorescence of tryptophan has been exploited in biochemistry to analyze protein structure and function. Several studies have demonstrated that the tryptophan content in proteins can be determined by measuring its fluorescence in proteins fully denatured with guanidine hydrochloride[4] or SDS and β-mercaptoethanol.[5] Yet, the fluorescence of tryptophan was only rarely exploited for total protein quantification.

In bottom-up proteomics, protein determination is the prerequisite for optimal protein digestion, and the quantification of the generated peptides is important for mass-spectrometry-based analysis. These measurements are particularly important in quantitative proteomics. Here we evaluate the spectral properties of tryptophan for determination of total protein and peptide contents. We show that the measurement of tryptophan fluorescence is a sensitive, reproducible, and convenient technique to quantify protein and peptide mixtures using either standard fluorescence spectrometers or microtiter-plate readers.

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■ EXPERIMENTAL SECTION

Preparation of Tissue Lysates. Mouse liver, brain, heart, and skeletal muscle were homogenized using a tissue blender (IKA Turrax) in lysis buffer containing 2% SDS, 0.1 M Tris-HCl, pH 7.8, and 50 mM DTT at a buffer to tissue ratio of 5. The homogenates were incubated in a boiling water bath for 5 min and, after chilling to room temperature, were clarified by centrifugation at 16 000 g for 5 min. Lysis of cells was carried out in a similar manner except that homogenization was substituted by sonication. For comparison of the tryptophan fluorescence (WF) assay with the BCA method, a tissue lysate was prepared without DTT. To study interference of various compounds on the WF and BCA methods, the lysate was prepared in a buffer containing 100 mM NaCl and 0.1 M Tris-HCl, pH 7.8. This tissues lysate was used directly in BCA, whereas for the WF assay the lysate was boiled for 5 min after addition of SDS and DTT, to final concentrations of 2% and 50 mM, respectively.

Preparation of Peptide Digests. Total peptides were obtained by processing the tissue lysates with the FASP method using Amicon Ultra 15 Ultracel 30k (Millipore) filters.[6] Briefly, an aliquot of whole brain lysate in SDS with DTT (see above) containing 5 mg of total protein was processed. SDS and DTT were depleted by consecutive washes with 8 M urea and 0.1 M Tris-HCl, pH 8.5, and the proteins were digested with 50 μg of trypsin. Peptides were eluted in 0.1 M Tris-HCl, pH 8.5.

Determination of the Tryptophan Content in Whole Lysates of Tissues and Cultured Cells. About 100 mg of cell pellets or pieces of mouse tissues were homogenized in 1 mL of 25 mM Tris-HCl, pH 7.8, using the above-mentioned tissue blender. To digest RNA, 50 units of RiboShredder (Epicentre, Madison, WI) were added and the suspensions were incubated for 30 min. Then, 120 μL of 0.1 M Tris-HCl, pH 7.8, buffer containing 25 mM $MgCl_2$, 5 mM $CaCl_2$, and 100 μg of DNase (DN25, Sigma, St. Louis) was added and the mixture incubated at 37 °C for 1h. After the DNA cleavage step, the suspensions were extracted with 1.2 mL of 2:1 (v/v) chloroform/methanol to remove lipids.[7] The methanol−water phase was collected and dialyzed against water overnight. Finally, the suspension was freeze-dried, and the residue was quantified gravimetrically. The residue was dissolved in 8 M urea containing 0.1 M Tris-HCl, pH 7.8, and its tryptophan content was determined by fluorescence spectrometry according to Payot.[4]

Fluorescence Spectrometry. Emission spectra of tryptophan were recorded using a Cary Eclipse Fluorescence Spectrometer (Varian) at 20 °C if not otherwise indicated. The fluorescence was excited at 295 nm, and the emission was recorded from 320 to 400 nm. The fluorescence of the whole cell lysates diluted in 8 M urea containing 10 mM Tris-HCl, pH 8.5, was measured in standard cuvettes (10 × 10 mm^2), whereas the measurements of peptide digests were conducted in semimicro cuvettes (4 × 4 mm^2) in 20 mM Tris-HCl, pH 8.5. Stocks of tryptophan and standards were prepared in 50% ethanol and were diluted before analysis. The concentration of the tryptophan and N-acetyl-tryptophan amide were assessed by absorption spectrometry on a double beam Cary 300Bio (Varian) instrument using ε_{280} = 5700 and 5500 M^{-1} cm^{-1}, respectively.

96-Well-Plate-Based WF Assay. Corning Costar 96-well black flat-bottomed polystyrene plates (Sigma-Aldrich, CLS3915) were used. The lysates and standards were directly spiked into 96-well plates prefilled with 100 or 200 μL of the adequate buffer. Tryptophan was used for the standard curve (1−100 μL spike-in), which was always measured concomitantly with the samples. The measurements of the tissue lysates were carried out in 200 μL of 8 M urea and 10 mM Tris-HCl, pH 8.5, where 1, 2, or 3 μL of lysates were added to the buffer. Peptide mixtures were quantified neat (100 μL in the buffer used for digestion) along with tryptophan standards in 100 μL of Tris-HCl, pH 8.5. The readout was the absolute tryptophan content expressed by mass. All experiments were carried out at least in triplicate (up to 12 replicates). Safire2 or Infinite 200 (Tecan) plate readers equipped with a fluorescence module were used. The excitation was set to 295 nm with a 5 nm bandwidth and the emission to 350 nm with a 20 nm bandwidth. Individual measurements consisted of 10 reads each with 50 μs integration time. The temperature was set to 25 °C. Before each measurement, orbital-type shaking was applied for 5 s followed by 2 s resting time.

Bicinchoninic Acid Assay. All protein determinations by means of the BCA were carried out using the Pierce BCA Protein Assay Kit (Thermo) in 96-well-plate format. The assay mixture contained 200 μL of the reagent (solution A + B) and 20 μL of sample containing either tissue lysates or BSA standard as well as different potential interfering reagents. Absorbance was read with 25 flashes at 562 nm using 9 nm bandwidth using an Infinite 200 plate reader.

■ RESULTS AND DISCUSSION

Tryptophan fluorescence is highly sensitive to its microenvironment with regard to proteins and to the polarity of the solvent. In addition, tryptophan fluorescence is quenched by several amino acids as well as many substances contained in buffers such as detergents. Temperature and pH are two further factors influencing the intensity of tryptophan fluorescence.[8] These effects were studied in detail using pure proteins or peptides in the past, demonstrating their high impact on the spectral properties of tryptophan.[4,9] However, most of these effects were minimized by unfolding proteins with guanidine HCl or SDS[4,5] and by controlling temperature and pH. In addition, the influence of the residues flanking tryptophan should be averaged when complex protein and peptide mixtures are analyzed.

In bottom-up proteomic workflows, the total protein content of the sample before proteolytic cleavage and the concentration of the resulting peptides have to be determined. The first measurement is important to optimize the digestion process, whereas the second allows for control of the yield of the protein cleavage process and adjustment of the peptide amount for mass spectrometry analysis.

Measurements of Tryptophan Fluorescence for Determination of Protein in Cell Lysates. Most commonly in proteomic analysis, protein solubilization is achieved by the addition of detergents. Because most detergents quench fluorescence at the concentration used for tissue lysis, we considered performing tryptophan quantification in a buffer containing 8 M urea. The spectra of both proteins and tryptophan had their maxima above 350 nm, indicating that the tryptophan indole moieties were freely exposed to the solvent (Figure 1A). Notably, even at relatively high concentrations of detergents, the tryptophan spectra were only a little affected, suggesting negligibly low interaction of detergents with the proteins under these conditions (Figure 1B). Both the

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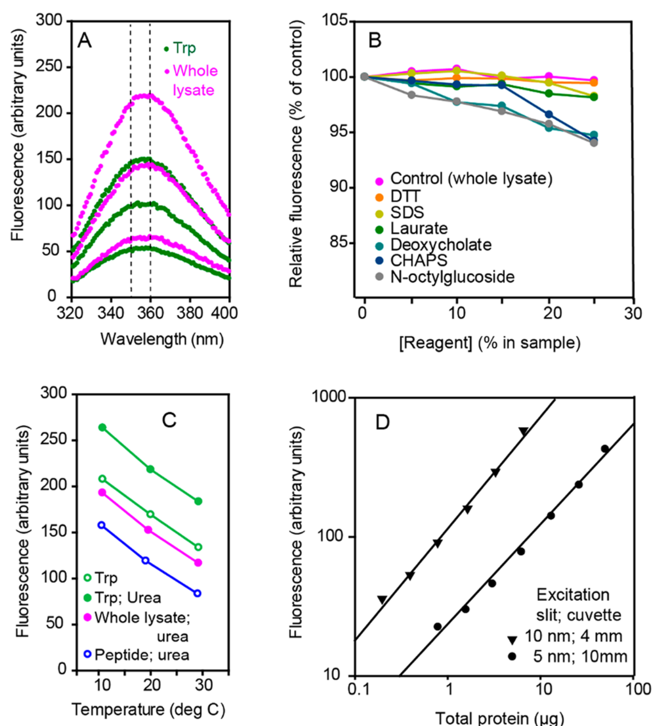

Figure 1. Spectral properties of free and intrinsic tryptophan. (A) Emission spectra of whole cell lysates (6, 12, and 18 μg of total protein) and pure tryptophan (0.05, 0.1, and 0.15 μg) in 2 mL of 8 M urea and 10 mM Tris-HCl, pH 7.8. (B) Quenching effect of detergents and DTT. Reagent concentration refers to concentration in 2 μL of solution added to 2 mL of 8 M urea. (C) Effect of temperature on the spectral intensities. Amounts of added per 2 mL were 0.05 μg of tryptophan, 10 μg of total protein of cell lysate, and 7.5 μg of peptide. (D) Fluorescence intensity versus protein amount measured in 8 M urea and 10 mM Tris-HCl, pH 7.8. Fluorescence was excited at 295 nm using excitation slits of 5 nm (D) and 10 nm (A–D). Emission was recorded from 320 to 400 nm. For the measurements, 10 × 10 mm^2 (A–D) and 4 × 4 mm^2 (D) cuvettes were used.

unchanged spectral maximum and intensity are the prerequisites for a quantitative assay.

Next, we analyzed the effect of temperature on the fluorescence intensity. Similar changes of 1.4−1.6% per degree were observed for tryptophan and protein lysates (Figure 1C). These results emphasize the importance of keeping the temperature constant during the measurements and demonstrate that the WF assay can be carried out at a wide range of temperatures.

Finally, we determined the range in which protein can be assayed by the fluorescence measurements in 8 M urea. Using a 10 nm bandwidth for the excitation slit, a linear correlation of protein amount to fluorescence was observed from 0.5 to 20 μg (Figure 1D). These values correspond to an addition of 1 μL of 0.5−20 mg/mL of protein solution. Thus, the range covers typical concentrations of protein in tissue or cell lysates. Because larger amounts of detergent-containing lysates can be also be measured, the WF assay is suitable for protein determination in diluted lysates below 0.1 μg/mL. By using larger cuvettes and/or smaller excitation slits, the assay range can be shifted to higher protein amounts (Figure 1D).

Determination of Peptide Concentration in Protein Digests. Determination of the total peptide concentration provides important information on the quality and reproducibility of the protein cleavage step and has an obvious impact on the performance of the liquid chromatography system and mass spectrometer. For this purpose, peptide concentrations can be assayed using conditions described above for proteins. However, often only small amounts of peptides are available, and assaying the peptide concentrations in this way would be accompanied by a partial loss of the sample. Therefore, we tested whether the peptide content can be measured directly on the neat sample using tryptophan as a standard.

We compared the spectral properties of the peptides obtained by digestion of whole lysates of both mouse tissues and HeLa cells with those of free tryptophan. We found that the spectra of the peptides dissolved in a buffer alone or the buffer with 8 M urea had maxima above 350 nm but were different in their intensities (Figure 2A). Under the same conditions, the spectra of free tryptophan showed similar changes in intensities (Figure 2A). The differences in the fluorescence intensities reflect the higher quantum yield of the indole moiety in urea than in water. The intensity of tryptophan fluorescence increases continuously with increasing urea concentrations by about 5% per (mole per liter) unit of urea concentration change (Figure 2B). Therefore, small differences in urea concentration across samples and standards have a negligibly low effect on the measured peptide or content. At pH values lower than 8.5, we observed a mutual decrease of the spectral intensities of the free and intrinsic tryptophan. In the pH range of 7.5−8.5, which is most commonly used for protein digestion with trypsin, endoproteinases LysC or GluC, in Tris or ammonium bicarbonate buffers, only small changes in the fluorescence intensities were observed (Figure 2C). In contrast, at low pH, the fluorescence decreased by 30−50%. Nevertheless, the fluorescence-based determination of peptide concentration in diluted acetic or formic acid is also possible.

Comparison of the ratio of the fluorescence intensities in buffers with and without urea varied only a little between free and the intrinsic tryptophan (Figure 2D). This indicates that the concentrations obtained using tryptophan as a standard for protein and peptide determination are very close together under both conditions.

Finally, we determined ranges of linearity of the fluorescence intensity in relation to peptide amount. Using 10 nm bandwidth for fluorescence excitation, we observed a linearity between 0.5 to 50 μg total peptide (Figure 2E). This range covers typical peptide concentrations in protein digests. A decrease of the excitation slit to 5 nm allows measurements of more concentrated peptides (Figure 2E).

Content of Tryptophan Varies Little between Tissues and Cells. We have been using the measurements of tryptophan fluorescence for many years.[10,11] However, the protein contents were calculated under the assumption that proteins contain on average 2.2% tryptophan by mass. This value reflects the 1.3% frequency of the tryptophan codon in human genes.[12] Because this value does not necessarily reflect the actual tryptophan content in mammalian proteins, we decided to assess its abundance experimentally. For this purpose, we determined tryptophan in four mouse tissues and three human cell lines. We prepared homogenates that were depleted of nucleic acid, lipids, and other small molecules. Nucleic acids were cleaved with RNase and DNase, lipids extracted according to the Folch procedure,[7] and the resulting water−methanol phase dialyzed against water. Subsequently, the dialysate was freeze-dried, and the residue was quantified gravimetrically then reconstituted in 8 M urea. The fluorescence measurements revealed similar tryptophan con-

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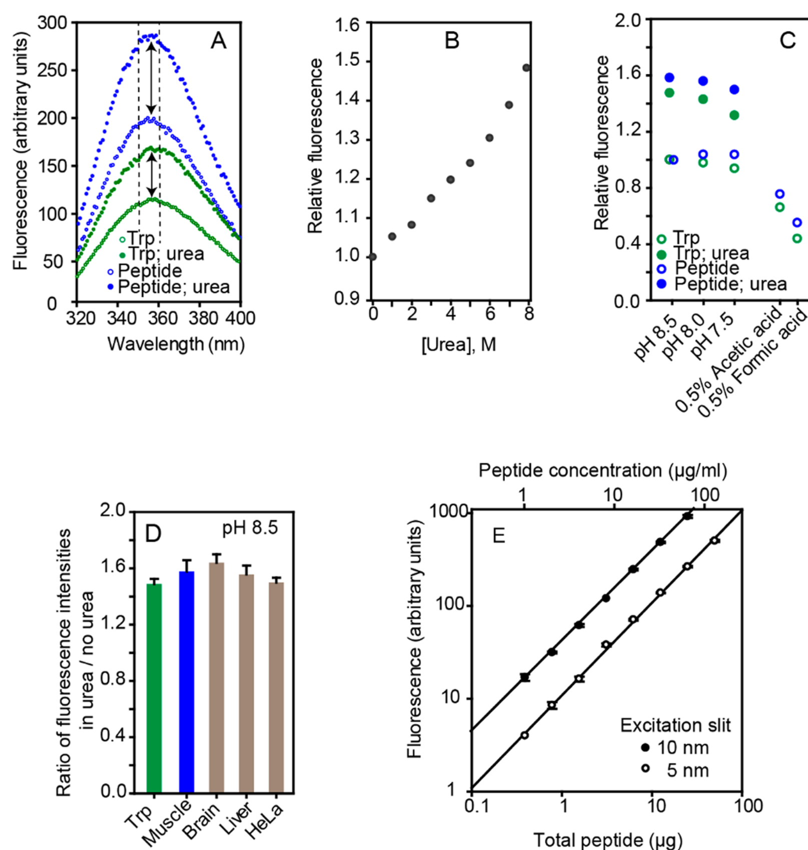

Figure 2. Fluorescence of free tryptophan and peptide mixtures. (A) Emission spectra of free (0.15 μg) and intrinsic tryptophan in peptides (17 μg) in the presence or absence of 8 M urea in 2 mL of 10 mM Tris-HCl, pH 7.8. (B) Dependence of tryptophan fluorescence on urea concentration. Measurement conditions were the same as in A. (C) Effect of pH on the fluorescence intensity of free tryptophan and a mixture of peptides obtained by digestion of the whole lysate of mouse muscle. Amounts of tryptophan and peptides used were the same as in A. (D) Ratio of the fluorescence intensity measured in the presence and absence of 8 M urea. (E) Fluorescence intensity versus peptide amount. Fluorescence was excited at 295 nm using excitation slits of 5 nm (A–E) and 10 nm (E). Emission was recorded from 320 to 400 nm. For the measurements, 4 × 4 mm^2 cuvettes were used.

tents across the analyzed tissues and cells (Table 1). Therefore, the mean value of 0.0117 g of tryptophan per gram of total protein can be used for the calculation of the protein content in mouse and human samples.

Finally, we validated the WF assay using BCA. We observed 5−10% higher values in BCA than in the WF assay (Table 1). Considering the possibility of up to several fold variation between different protein assays with respect to the amino acid composition of standard proteins,[1] this difference appears rather small.

Table 1. Content of Tryptophan in Tissues and Cells and Comparison of the Total Protein Determination Using WF and BCA Methods

tissue/cells	tryptophan content by biochemical analysis (% by weight)	relative protein concentration WF/BCA[a]
liver	1.07	0.86 ± 0.06
brain	1.19	0.96 ± 0.02
heart	1.11	0.94 ± 0.06
skeletal muscle	1.25	0.90 ± 0.02
HCT116	1.19	0.99 ± 0.04
HepG2	1.25	0.97 ± 0.02
A549	1.13	0.92 ± 0.06
average	1.17 ± 0.07	0.93 ± 0.04

[a]BSA was used as the standard.

Adaptation of the WF Assay for Use in 96-Well Format. Nowadays, fluorimeters are infrequently used, and the cleaning of a cuvette between samples can be considered cumbersome. Therefore, we sought to adapt the assay to a plate-reader format, thus enabling a higher throughput. After determining optimal measuring parameters, the linearity of the method was assessed with tryptophan standards in either buffered urea or a simple buffer. Linearity was given up to 5 μg and down to 0.01 μg, which was set as the lower limit of quantitation for this assay (Figure 3A,B). No significant difference in linearity was observed when 100 or 200 μL buffered urea per well was used to spike in the tryptophan standards (Figure 3C). As expected, the coefficient of variation was higher for those samples below the limit of detection (Figure 3D). The same held true for protein lysates and digest samples when plotting their protein concentration (Figure 3E). Typically, 1, 2, or 3 μL of tissue lysate were spiked in 200 μL of buffered urea. Although the addition of 3 μL of lysate tended toward the upper limit of linearity, 1 μL was more variable because of the little-pipetted amount. Therefore, a spike-in volume of 2 μL is our recommendation for this assay when using whole cell tissue lysates. Peptides (100 μL per well) were measured neat in their digestion buffer (0.1 M Tris-HCl, pH 8.5). Generally, the limit of quantitation of 0.01 μg tryptophan

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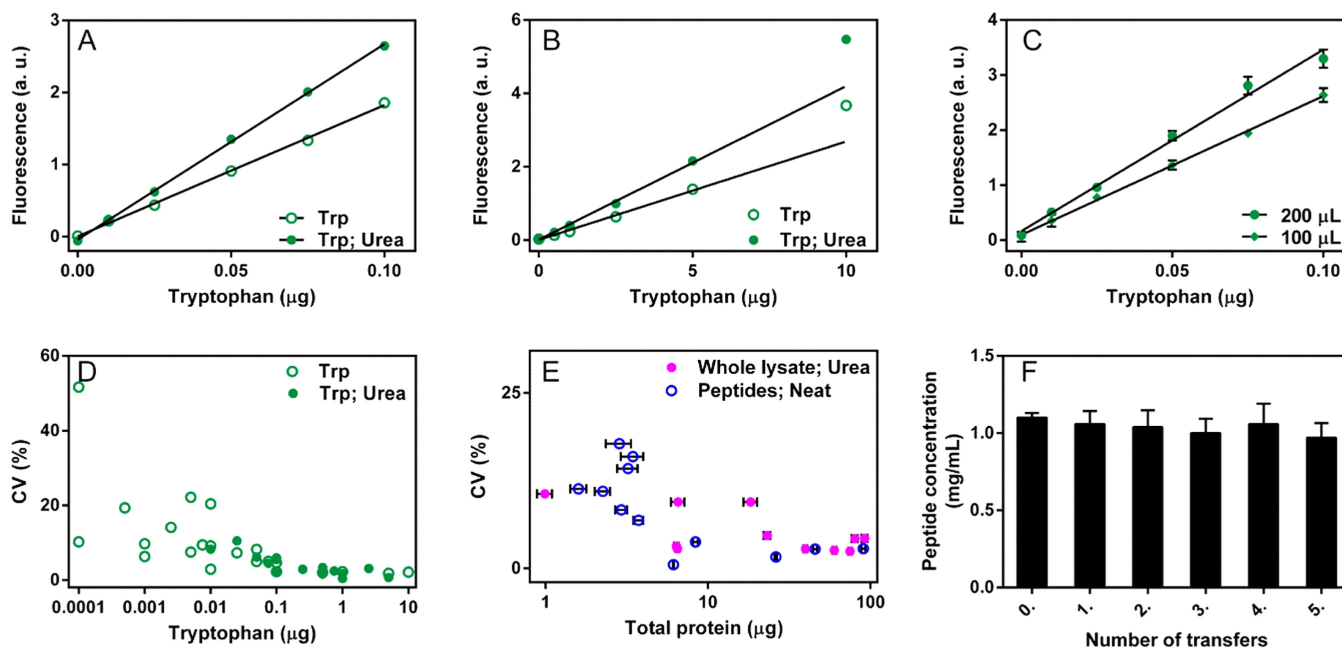

Figure 3. Protein determination in the 96-well format. (A and B) Linearity of fluorescence intensity of tryptophan standards (0.0001−10 μg). (A) Lower limit; Trp in 8 M urea and 0.01 M Tris-HCl, pH 8.5, or in 0.01 M Tris-HCl, pH 8.5. (B) Higher range; 10 μg data points were excluded from the linear regression. (C) Trp in 200 or 100 μL 8 M urea and 0.01 M Tris-HCl, pH 8.5. (D) Coefficient of variation versus tryptophan content of tryptophan standards (0.0001−10 μg) in 8 M urea and 0.01 M Tris-HCl, pH 8.5, or in 0.01 M Tris-HCl, pH 8.5. (E) Coefficient of variation versus protein concentration of lysates (in 8 M urea and 0.01 M Tris-HCl, pH 8.5) and peptides (neat). (F) Recovery of neat peptides from polystyrene black plates. Average and standard deviations are depicted for each experiment.

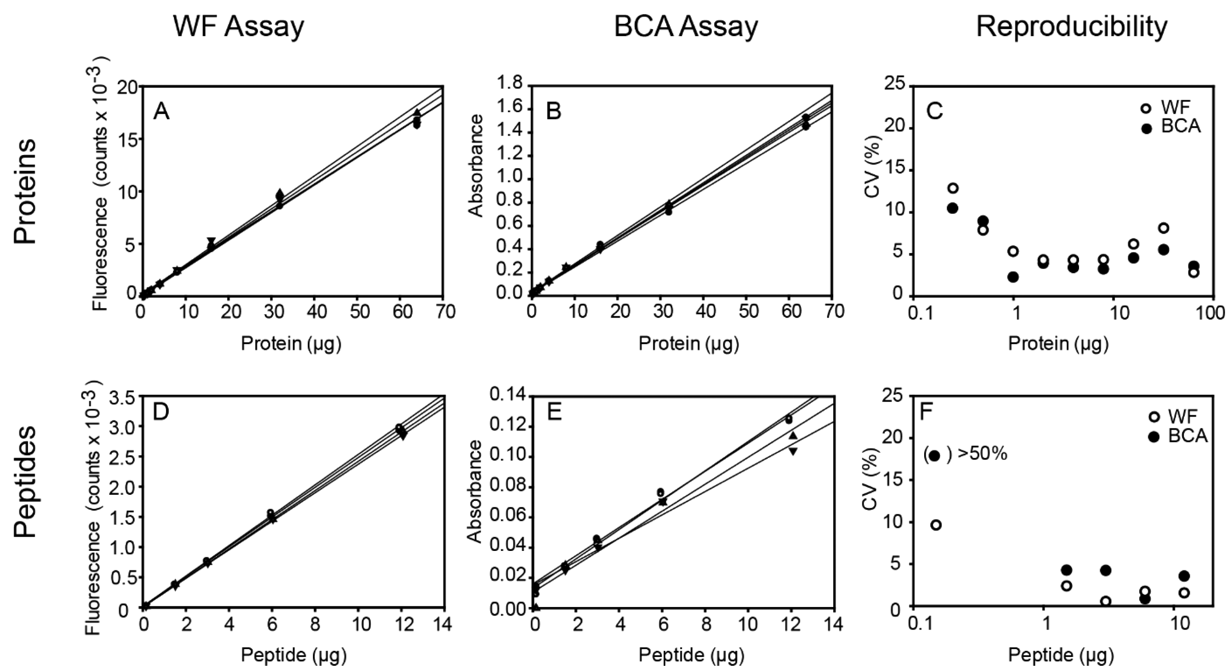

Figure 4. Comparison of sensitivity and robustness of the WF and BCA methods in the 96-well-plate formats. Aliquots of whole mouse brain lysate containing (A−C) 0.25−64 μg of total proteins or (D−F) tryptic digest containing 0.1−12 μg of peptide were assayed with WF (A and D) and BCA (B and E) methods. Each measurement was repeated 4 times. The CV values in C and F were calculated using data from A and B and from D and E, respectively. The whole cell lysate was prepared in 2% SDS and 0.1 M Tris-HCl, pH 7.8, whereas the digests were obtained by tryptic cleavage of mouse brain lysate using the FASP method (see Experimental Section).

per well, corresponding to 0.87 μg protein, was verified both for lysates and peptides, either in buffered urea or in the digestion buffer.

The recovery of neat peptides from the polystyrene 96-well plates was assessed. Adsorption was found negligible after one transfer step, allowing the recovery of all peptides for subsequent mass spectrometric analysis (Figure 3F).

Comparison of the WF and BCA Methods. The BCA method is probably the most frequently used method for total protein determination. We compared the sensitivity and

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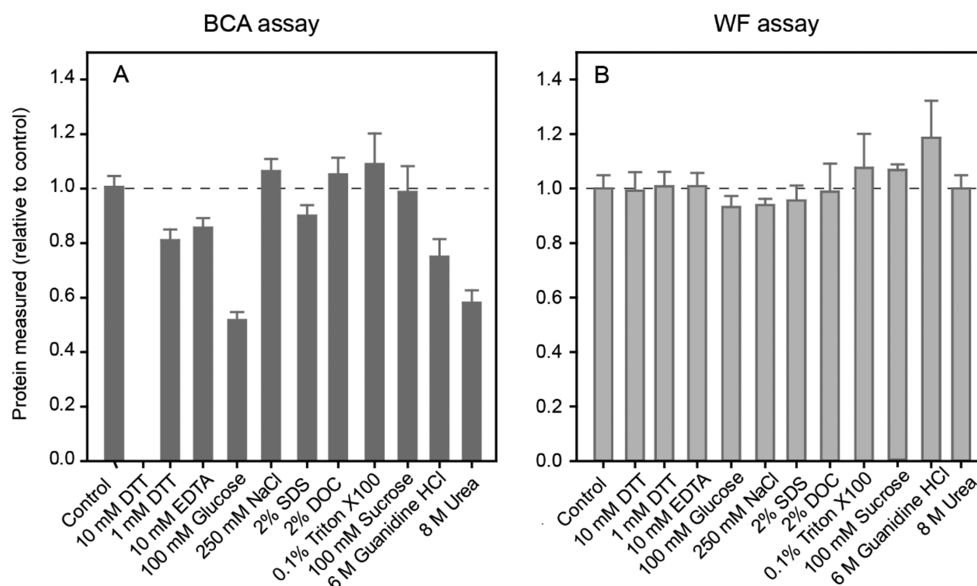

Figure 5. Effect of selected compounds on the WF and BCA methods. Aliquots (16 μL each) containing 10 μg of total protein in solutions of the assayed compounds were mixed with (A) 200 μL of BCA reagent or (B) 8 M urea and 0.01 M Tris-HCl, pH 8.5. BSA and tryptophan were used as standards for the BCA and WF methods, respectively. Values were corrected for blanks with interfering compounds. Each experiment was repeated 5 times.

reproducibility of the BCA method with those of our WF assay. We found that both methods have similar sensitivity and reproducibility for protein determination in tissue lysate (Figure 4 A−C). In the determination of peptide contents in protein digests, the WF assay appears to be more reproducible than the dye-based assay (Figure 4 E,F). The CV values for protein and peptide amounts below one microgram increase up to 10%. It is arbitrary whether such values are acceptable for determination of total protein and peptides. In 96-well format, any volume that forms a uniform layer in the well is suitable for determination.

Next, we compared the interference of selected reagents that are commonly used for sample preparation and those that potentially can be present in the lysed cells, for example, glucose in cell culture and blood plasma (Figure 5). Protein determination by BCA was significantly affected in the presence of free DTT, EDTA, glucose, guanidine hydrochloride, and urea (Figure 5A). In contrast, of all reagents tested, only guanidine hydrochloride has a significant effect on the WF assay (Figure 5B).

CONCLUSIONS

We present the rationale for a method for the total protein and total peptide determination that we have been using for several years. An important innovation present in this paper is the demonstration that the tryptophan-fluorescence-based assays can be carried out in a 96-well format, thus meeting demands of high-throughput analysis. We show that the direct quantitation of tryptophan by measuring its fluorescence is an easy, fast, and reliable way to quantify total protein and total peptide. The sensitivity of the WF assay for whole lysates is as good as that of the BCA method, whereas our assay is more reliable for quantitation of low amounts of peptides. The determination of total proteins requires only regular-quality urea as reagent, but quantitation of peptides does not need any chemicals and can be carried out directly in buffers used for protein cleavage. Notably, the peptide sample can be recovered after the measurement and used for mass spectrometric analysis. The WF assay is a direct measurement and does not consume time for color development as the BCA and other colorimetric assays do.

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Notes

The authors declare no competing financial interest.

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