Estimation of peptide concentration by a modified BCA assay

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Abstract

Although biuret based protein assays are theoretically applicable to peptide measurement, there is a high level of inter-peptide variation, determined largely by peptide hydrophobicity. This variation in peptide reactivity can be significantly reduced by heat-denaturation of peptides at 95 °C for 5 minutes in the presence of 0.1 M NaOH containing 1% (w/v) SDS, prior to incubation for 30 min at 37 °C in BCA standard working reagent. This modification to the standard bicinchoninic acid (BCA) assay protocol allows for an accurate, rapid and economical estimation of the peptide concentration within an unknown sample.

Key words: Assay; BCA; bicinchoninic acid; estimation; measurement; peptide.

There are a wide range of methods for estimating protein concentration, driven by the rise in proteomics/peptidomics over the last few years, but fewer approaches are available for quantifying the peptide content of a sample. Measurement of UV absorbance is often recommended however absorbance at 280 nm is influenced by frequency of tyrosine and tryptophan (as well as nucleotide and nucleic acid contamination), whilst absorbance at 210 nm is complicated by other organic compounds [1]. A number of fluorescent and luminescent approaches to peptide quantification are in use [2,3,4], however, the majority of these methods either react with the peptide amino-terminus or are biased towards certain amino acids within the sequence, and are frequently sensitive to photo-bleaching. Colorimetric protein assays, including the biuret-based Lowry [5] and bicinchoninic acid (BCA) [6] assays are occasionally utilised

to quantify peptide [7,8], though neither is recommended for this application. Currently, there are no convenient published colorimetric methods for peptide quantification. In the present study we demonstrate a modification which improves the applicability of the BCA assay to quantification of the peptide concentration in a sample.

A bicinichoninic acid (BCA) protein assay kit and glycerol were supplied by Fisher Scientific (UK). Falcon™ 96-well plates were supplied by BD Biosciences (USA). SDS was supplied by Melford (UK). The phosphopeptides and non-phosphorylated counterparts (hydrophilic scores given in brackets) ALR(pS)NFERI (0.3), SQKGQESE(pY) (1.1/0.9), TVEAVA(pY)APK (0.1/-0.1), and YI(pS)PLKSPY (-0.5) were synthesised by Pepceuticals (UK). Peptides VIVHSATGFKQSSKALQRPVASDFEPQ (0.0), LYGFNLVIVHSATGFKQSSK (HS: -0.5), VHSATGFKQSSKALQRPVASD (0.2) and QAGILARNLUPMVATVQGQN (-0.4) were supplied by Alta Biosciences (UK). Peptide LSRH (0.3) was synthesised at Nottingham Trent University. Predicted hydrophilic scores were determined using the *Peptide Property Calculator* at Innovagen (http://www.innovagen.se/). Rabbit muscle aldolase and bovine liver catalase was supplied by GE Healthcare (UK). All other reagents were procured from Sigma-Aldrich (UK). Peptide stock solutions were dissolved in DMSO to 10 mg/ml. Cell lines (JY and T2) were cultured in RPMI 1640 + 10% (v/v) FCS, 20 mM L-glutamine at 37 °C in a 5% CO_2 atmosphere, and subcultured according to standard protocols when in log-phase growth and 80% confluency.

Standard method: Fifty microlitres of sample was mixed with 50 µl of 0.2 M NaOH containing 2% (w/v) SDS in 0.5 ml microfuge tubes, and heated to 95 °C for 5 minutes using a Biometra Uno Thermoblock (Germany). After cooling to room temperature 25 µl of this sample mixture was transferred to a microplate (in triplicate) followed by 200 µl of BCA standard working reagent (SWR) (prepared by mixing BCA reagent A with reagent B in a 50:1 ratio) [6]. The microplate was covered (sealing film: SLS, UK), incubated at 37 °C for 30 minutes and the absorbance read at 570 nm (microplate reader: model 680 Bio-Rad, UK).

A 700-watt microwave oven may be used as an alternative to the 37 °C incubation step in the BCA assay. Prior to heating in the microwave, the microplate wells which did not contain any sample were filled with 200 µl of water (to prevent sample boiling and reduce inter-plate variation). The microplate was covered with sealing film and placed on the edge of the microwave's revolving

tray for two concurrent 15-second intervals. To reduce the intra-plate heating variation the microplate was manually rotated by 180 degrees after the first incubation period. Following microwave-incubation, the microplates were cooled to room temperature and the absorbance read as above.

Standard proteins (aldolase, BSA, casein and catalase: 5 mg/ml) in 20 mM Tris/HCl pH 8.0 were digested by incubation with 1% (w/w) trypsin for 72 hours at 37 °C in a orbital incubator (SI50, Stuart, UK). The undigested protein was removed by the addition of TCA to a final concentration of 7.2% (w/v) [9], followed by centrifugation at 10,000g for 20 minutes. After the supernatant was diluted by 1:10 in 0.1 M NaOH containing 1% (w/v) SDS, the peptide content of the supernatant was measured (as above).

Guinea pig liver (10.0 g) was homogenised in 100 ml of 50 mM Tris/HCl using a Waring Blender and clarified by centrifugation at 20,000g for 30 minutes (Allegra X-15R, Beckman UK). Supernatant proteins were precipitated by ammonium sulphate (80% saturation) with stirring at 4 °C for 4 hours. The precipitate was collected by centrifugation (10,000g for 45 minutes at 4 °C), then dissolved and dialysed overnight against a 10 mM trimethylamine-HCl pH 8.0 buffer. The protein concentration was determined [6] and 0.5 g was tryptically digested as above. Undigested material was precipitated by the addition of 9 volumes of ice-cold acetone and incubated overnight at -20 °C, then centrifuged at 10,000g for 20 minutes at 4 °C. The peptide supernatant was dried using inert gas (N_2) in a glass container. Peptide fragments were prepared from BSA and two lymphoblastic cell lines (JY and T2) using a similar method. After drying, peptides from digests were weighed, redissolved in DMSO and assayed by the modified peptide BCA (as above).

Figure 1 illustrates that linear and reproducible standard curves may be produced from tryptic digests of protein extracted from guinea pig liver, two lymphoblastic cell lines (JY and T2) and of BSA. We have found that using a standard BCA protocol [6] to quantify peptides in solution [7,8] produced a wide variation in inter-peptide reactivity (see Table 1). This was largely dependent upon two variables; peptide length and hydrophilic score, which together amount to a Spearman's rank correlation of 0.785 ($P < 0.01$) with BCA reactivity. In order to minimise this variation, a range of solubilisation reagents were tested including the addition of up to 5% (w/v) SDS, 10% (v/v) methanol, 0.15% (w/v) deoxycholate, 100 mM Na_2HPO_4, 0.1 M NaOH, and various combinations thereof

(data not shown). Table 1 demonstrates that solubilising the peptides at 0.5 mg/ml in 0.1 M NaOH containing 1% (w/v) SDS (coupled with heating at 95 °C for 5 minutes) reduced intra-peptide variability by 32% for the synthetic peptides tested and by 52% if the assay was restricted to nonamers and decamers (statistically significant to $P < 0.01$ using student T-test carried out on pooled SD values of assays performed using SDS/NaOH and water). Similar patterns were observed for tryptic digests of standard proteins and of cell lysates.

A range of incubation times was examined (data not shown), the recommended optimum incubation of 30 minutes using SWR of a 50:1 reagent A:B ratio was sufficient for the concentration range examined. Nevertheless, for measurement of low abundance peptides, alteration of the BCA SWR [6] to include a higher concentration of $CuSO_4$, or longer incubation periods may prove fruitful. Figure 1 illustrates standard calibration curves produced from tryptic digests of biological material are linear between 0.01 and 1.0 mg/ml, similar results were observed with both the synthetic peptides and protein digests tested. The alteration to the BCA method had no impact on the stability of the reaction, as with the standard assay there is little upwards drift in colour intensity once the plate is removed from incubation at 37 °C.

The use of microwave-incubation [10] allows for a rapid assay method, however inter-well variability was slightly higher than the standard 37 °C incubation (data not shown). Additionally, the microwave-incubation was not sufficient to replace the peptide denaturing step, as microwave-incubation of non-denatured samples in the presence of 0.1 M NaOH containing 1% (w/v) SDS only reduced inter-peptide BCA variation by 5% (similar to an unheated control: data not shown).

These modifications to the BCA assay allow for the rapid and accurate quantification of a sample's peptide concentration with standard laboratory reagents and equipment. The methods outlined above demonstrate that peptides may be assayed following precipitation of contaminating proteins by solvents or TCA. However, whilst the BCA assay carries a high degree of flexibility, it does have minor drawbacks which can be avoided by appropriate cleanup procedures. As the full compatibilities of the modified assay are yet to be determined it is recommended that an appropriate standard peptide or protein digest curve is prepared in the same matrix as samples.

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Figure 1: Peptide BCA standard graphs were produced from peptides derived from the tryptic digests of BSA, guinea pig liver protein and two cell lines (JY and T2) as per materials and methods. Peptides in DMSO were diluted in 0.1 M NaOH containing 1% (w/v) SDS prior to heating at 95 °C for 5 min and a BCA assay carried out in a microplate. The samples were then read at 570 nm.

Table 1: Mean absorbance (at 570 nm) and standard deviation (±) of synthetic and tryptically derived peptide populations assayed by BCA assay with or without denaturing in 1 % (w/v) SDS in 0.1 M NaOH. See materials for synthetic peptide sequences. Standard protein digests: peptides from aldolase, BSA, casein and catalase. Biological material digests: peptides from JY and T2 cell lines and a guinea pig liver homogenate.

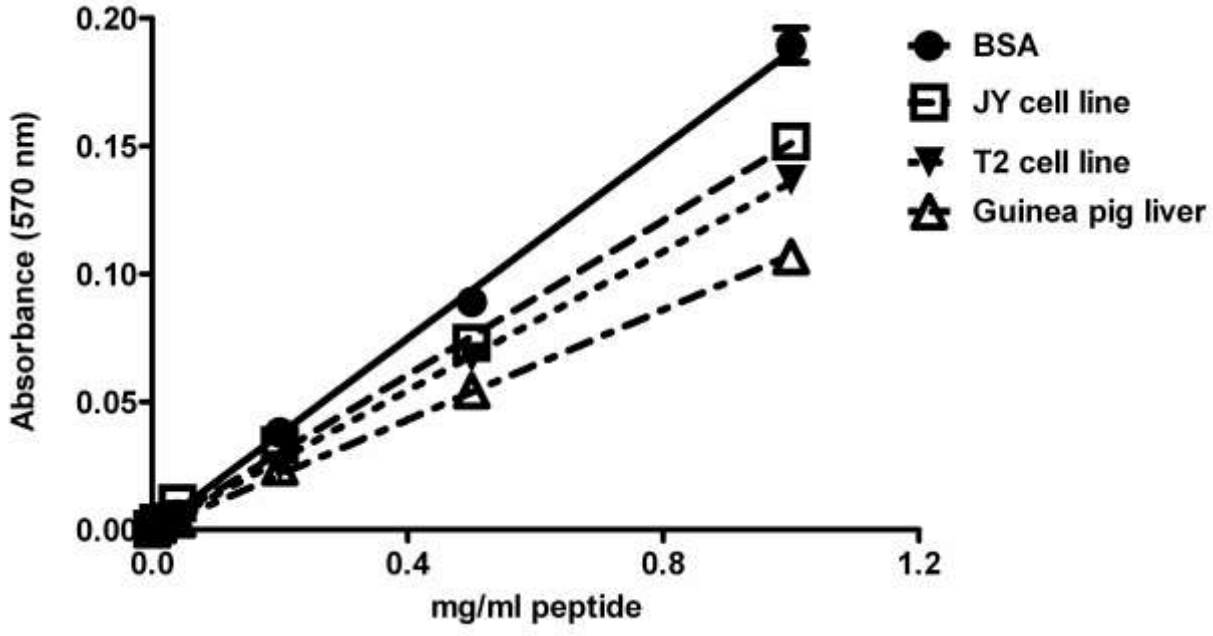
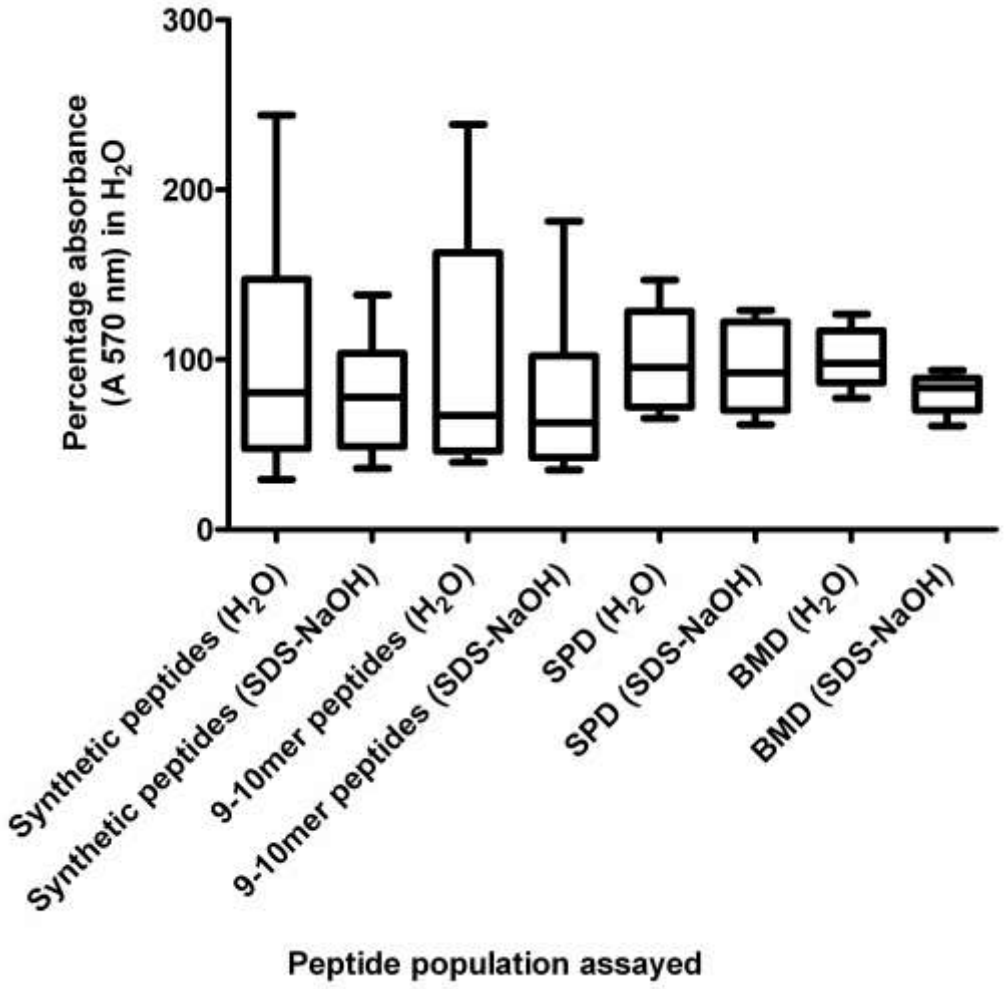


Figure 1

Figure 2


300
200
100
0
Percentage absorbance (A 570 nm) in H_2O
Synthetic peptides (H_2O)
Synthetic peptides (SDS-NaOH)
9-10mer peptides (H_2O)
9-10mer peptides (SDS-NaOH)
SPD (H_2O)
SPD (SDS-NaOH)
BMD (H_2O)
BMD (SDS-NaOH)
Peptide population assayed