УДК 547.466/.96+615.071

A.S. Kozhamzharova, L.S. Kozhamzharova,* S.O. Ordabekov, Z.B. Yesimseiitova**
Asfendiyarov Kazakh National Medical University
**M.Kh. Dulaty Taraz State University*
*** al-Farabi Kazakh National University*

THE QUALITATIVE AND QUANTITATIVE IDENTIFICATION OF AMINO ACIDS, POLYPEPTIDES AND PROTEINS THROUGH BIOCHEMICAL METHODS

This article has information about the biuret assay, epy method has several advantages, including speed, similar color development with different proteins, and few interfering substances. Its primary disadvantage is its lack of sensitivity.
Keywords: *proteins, Biuret, spectrophotometric, amino acids, micrograms, carboxylic acid.*

Amino acids are building blocks of all proteins, and are linked in series by peptide bond (-CONH-) to form the primary structure of a protein. Amino acids possess an amine group, a carboxylic acid group and a varying side chain that differs between different amino acids. There are 20 naturally occurring amino acids, which vary from one another with respect to their side chains. Their melting points are extremely high (usually exceeding 200°C), and at their pH, they exist as zwitterions, rather than as unionized molecules. Amino acids respond to all typical chemical reactions associated with compounds that contain carboxylic acid and amino groups, usually under conditions where the zwitter ions form is present in only small quantities. All amino acids (except glycine) exhibit optical activity due to the presence of an asymmetric α – Carbon atom. Amino acids with an L – configuration are present in all naturally occurring proteins, whereas those with D – forms are found in antibiotics and in bacterial cell walls. Certain functional groups in amino acids and proteins can react to produce characteristically coloured products. The colour intensity of the product formed by a particular group varies among proteins in proportion to the number of reacting functional or free groups present and their accessibility to the reagent. Now we will discuss various colour-producing reagents (dyes) to qualitatively detect the presence of certain functional groups in amino acids and proteins.

Biuret reaction for identification of peptide bonds.

When substances containing two or more peptide bonds react with the biuret reagent, alkaline copper sulfate, a purple complex is formed. The colored product is the result of coordination of peptide nitrogen atoms with The amount of product formed depends on the concentration of protein. In practice, a calibration curve must be prepared by using a standard protein solution. The very best protein to use as a standard is a purified preparation of the protein to be assayed. Since this is rarely available, the experimenter must choose another protein as a relative standard. A relative standard must be selected that provides a similar color yield. An aqueous solution of bovine serum albumin (BSA) is a commonly used standard. Various known amounts of this solution are treated with the biuret reagent, and the color is allowed to develop. Measurements of absorbance at 540 nm are made against a blank containing biuret reagent and buffer or water. The A540 data are plotted versus protein concentration (mg/mL) or amount of protein (mg). Unknown protein samples are treated with biuret reagent and the is measured after color development. The protein concentration is determined from the standard curve. The biuret assay has several advantages, including speed, similar color development with different proteins, and few interfering substances. Its primary disadvantage is its lack of sensitivity.

The biuret test for proteins positively identifies the presence of proteins in solution with a deep violet colour. Biuret, $H_2NCONHCONH_2$, reacts with copper (II) ions in a basic solution to form a deep violet complex. The peptide linkages in proteins resemble those in biuret and also form deep violet complexes with basic copper (II) ions in solution. The general or biuret complex formed between the protein linkages and the copper (II) ion of the biuret test is shown in following figure.

Table 1 - Methods of protein measurement

Method	Sensitivity	Time	Principle	Interferences	Comments
Biuret	Low 1–20 mg	Moderate 20–30 min	Peptide bonds alkaline purple complex Cu2+ :	Zwitterionic buffers, some amino acids	Similar color with all proteins. Destructive to protein samples.
Lowry	High ~5 mg	Slow 40–60 min	1) Biuret reaction (2) Reduction of phosphomolybdatephosphotungstate by Tyr and Trp	Ammonium sulfate, glycine, zwitterionic buffers, mercaptans	Time-consuming. Intensity of color varies with proteins. Critical timing of procedure. Destructive to protein samples.
Bradford	High ~1 mg	Rapid 15 min	Lmax of Coomassie dye shifts from 465 nm to 595 nm when proteinbound	Strongly basic buffers; detergents Triton X-100, SDS	Stable color that varies with proteins. Reagents commercially available. Destructive to protein samples. Discoloration of glassware.
Spectrophotometric (*A* 280)	Moderate 50 - 1000 mg	Rapid 5–10 min	Absorption of 280-nm light by aromatic residues	Purines, pyrimidines, nucleic acids	Useful for monitoring column eluents. Nucleic acid absorption can be corrected. Nondestructive to protein samples. Varies with proteins.

The reaction takes its name “Biuret Reaction” from the fact that biuret itself, obtained by heating urea, gives a similar colored complex with cupric ions.

Reagents:

1,0% wheat protein
1,0% egg protein
0,1% solution of amino acids
10,0% sodium hydroxide
0,1% copper sulfate

Procedure:

Prepare three test tubes:
1 test tube: add 0,2 ml of 1,0% wheat protein
2 test tube: add 0,2 ml of 1,0% egg protein
3 test tube: add 0,2 ml of 0,1 % solution of amino acids
To each of the test tube, add 0,2 ml of 10,0 % sodium hydroxide and 1-2 drops of 0,1 % copper sulfate. Mix the mixtures. As a result of the reaction a violet-coloured compound is formedin 1 and 2 test tubes.

The Spectrophotometric Assay (Absorbance assay 280 nm)

Most proteins have relatively intense ultraviolet light absorption centered at 280 nm. This is due to the presence of aromatic tyrosine and tryptophan residues in the protein. However, the amount of these amino acid residues varies in different proteins, as was pointed out earlier. If certain precautions are taken, the A 280 value of a protein solution is proportional to the protein concentration. In practice, the procedure is simple and rapid. A protein solution is transferred to a quartz cuvette and the A 280 is read against a reference cuvette containing the protein solvent only (buffer, water, etc.). For the average protein, a concentration of 1 mg/mL will yield an absorbance at 280 nm of about 1. If the extinction coefficient is known for the protein, its exact concentration may be calculated from the absorbance at 280 nm. Cellular extracts contain many other compounds that absorb in the vicinity of 280 nm. Nucleic acids, which can be common contaminants in a protein extract, absorb strongly at 280 nm (lmax = 260). Early researchers developed a method to correct for this interference by nucleic acids. Mixtures of pure protein and pure nucleic acid were prepared, and the ratio A 280\260 was experimentally determined. The following empirical equation may be used for protein solutions containing up to 20% nucleic acids.

Although the spectrophotometric assay of proteins is fast, relatively sensitive, and requires only a small sample size, it is still only an estimate of protein concentration unless its extinction coefficient is known. It has certain advantages over the colorimetric assays in that most buffers and ammonium sulfate do not interfere and the procedure is nondestructive to protein samples. The spectrophotometric assay is particularly suited to the rapid and continuous
measurement of protein elution from a chromatography column, where only protein concentration *changes* are required [9].

Principle: proteins in solution absorb ultraviolet light with absorbance maxima at 280nm and 200 nm. Amino acids with aromatic range are the primary reason for the absorbance peak at 280 nm. Peptide bonds are primarily responsible for the at 200 nm. Secondary, tertiary, and quaternary structure all affect absorbance, therefore factors such as pH, ionic strength, etc can alter th absorbance spectrum.

Equipment: In addition to standard liquid handling supplies a spectrophotometer with UV lamp and quartz cuvette are required.

Procedure: Carry out steps 1-4 (280 nm only) for a very rough estimate. Carry out all steps if nucleic acid contamination is likely.

Warm up the UV lamp (about 15 min.)
Adjust wavelength to 280 nm
Calibrate to zero absorbance with buffer solution only
Measure absorbance of the protein solution
Adjust wavelength to 260 nm
Calibrate to zero absorbance with buffer solution only
Measure absorbance of the protein solution
Analysis

Unknown proteins or protein mixtures. Use the following formula to roughly estimate protein concentration. Path length for most spectrometers is 1 cm.

Concentration (mg/ml) = Absorbance at 280 nm divided by path length (cm.)

Pure protein of known absorbance coefficient. Use the following formula for a path length of 1 cm. Concentration is in mg/ml, %, or molarity depending on which type coefficient is used.

concentration = Absorbance at 280 nm divided by absorbance coefficient

To convert units, use these relationships:

Mg protein/ml = % protein divided by 10 = molarity divided by protein molecular weight

Unknowns with possible nucleic acid contamination. Use the following formula to estimate protein concentration:

Concentration (mg/ml) = (1.55 x A280) - 0.76 x A260)

The Bradford Assay

The many limitations of the biuret and Lowry assays have encouraged researchers to seek better methods for quantitation of protein solutions. The **Bradford assay,** based on protein binding of a dye, provides numerous advantages over other methods. The binding of Coomassie Brilliant Blue dye to protein in acidic solution causes a shift in wavelengh of maximum absorption of the dye from 465 nm to 595 nm. The absorption at 595 nm is directly related to the concentration of protein. The dye reagent interacts primarily with positively-charged (basic) amino acid residues (arginyl, lysyl, and histidyl) in proteins. In addition, there are probably weaker interactions between the dye molecules and hydrophobic, aromatic amino acid residues (tyrosinyl, phenylalanyl, and tryptophanyl). Proteins that have more than an average number of these interacting residues show higher sensitivity and thus higher absorbance values at 595 nm.

In practice, a calibration curve is prepared by plotting of standard protein versus absorbance at 595 nm, which is then used to estimate the amount of protein in unknown samples. Recommended standard proteins include bovine gamma globulin (IgG), lysozyme, and ovalbumin because they have closer to the average or typical number of amino acid residues that bind the dye (see above). Although BSA is often recommended in the literature, it is not a good choice for a standard for the Bradford assay, as it has a high content of reacting amino acid
residues. The 595 nm absorbance of BSA is about 2.1 times that of IgG (Figure 3.6).

The assay requires only a single reagent, an acidic solution of Coomassie Brilliant Blue G-250. After addition of dye solution to a protein sample, color development is complete in 2 minutes and the color remains stable for up to 1 hour. The sensitivity of the Bradford assay rivals and may surpass that of the Lowry assay. With a microassay procedure, the Bradford assay can be used to
determine proteins in the range of 1 to The Bradford assay shows significant variation with different proteins, but this also occurs with the Lowry assay and can be avoided by use of the proper protein standard. The Bradford method not only is rapid, but also has very few interferences by nonprotein components. The only known interfering substances are the detergents Triton X-100 and sodium dodecyl sulfate. The many advantages of the Bradford assay have led to its wide adoption in biochemical research laboratories.

The Bradford assay is very fast and uses about the same amount of protein as the Lowry assay. It is fairly accurate and samples that are out of range can be retested within minutes. The Bradford is recommended for general use, especially for determining protein content of cell fractions and assesing protein concentrations for gel electrophoresis.

Assay materials including color reagent, protein standard, and instruction booklet are available from Bio-Rad Corporation. The method described below is for a 100 µl sample volume using 5 ml color reagent. It is sensitive to about 5 to 200 micrograms protein, depending on the dye quality. In assays using 5 ml color reagent prepared in lab, the sensitive range is closer to 5 to 100 µg protein. Scale down the volume for the "microassay procedure," which uses 1 ml cuvettes. Protocols, including use of microtiter plates are described in the flyer that comes with the Bio-Rad kit [8].

Principle

The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay

is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range.

Equipment

In addition to standard liquid handling supplies a visible light spectrophotometer is needed, with maximum transmission in the region of 595 nm, on the border of the visible spectrum (no special lamp or filter usually needed). Glass or polystyrene (cheap) cuvettes may be used, however the color reagent stains both. Disposable cuvettes are recommended.

Reagents

Bradford reagent: Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol, add 100 ml 85% (w/v) phosphoric acid. Dilute to 1 liter when the dye has completely dissolved, and filter through Whatman #1 paper just before use.

(Optional) 1 M NaOH (to be used if samples are not readily soluble in the color reagent).

The Bradford reagent should be a light brown in color. Filtration may have to be repeated to rid the reagent of blue components. The Bio-Rad concentrate is expensive, but the lots of dye used have apparently been screened for maximum effectiveness. "Homemade" reagent works quite well but is usually not as sensitive as the Bio-Rad product.

Assay

Warm up the spectrophotometer before use.

Dilute unknowns if necessary to obtain between 5 and 100 µg protein in at least one assay tube containing 100 µl sample

If desirred, add an equal volume of 1 M NaOH to each sample and vortex (see Comments below). Add NaOH to standards as well if this option is used.

Prepare standards containing a range of 5 to 100 micrograms protein (albumin or gamma globulin are recommended) in 100 µl volume. Add 5 ml dye reagent and incubate 5 min.

Measure the absorbance at 595 nm.

Analysis

Prepare a standard curve of absorbance versus micrograms protein and determine amounts from the curve. Determine concentrations of original samples from the amount protein, volume/sample, and dilution factor, if any.

Comments

The dye reagent reacts primarily with arginine residues and less so with histidine, lysine, tyrosine, tryptophan, and phenylalanine residues. Obviously, the assay is less accurate for basic or acidic proteins. The Bradford assay is rather sensitive to bovine serum albumin, more so than "average" proteins, by about a factor of two. Immunoglogin G (IgG - gamma globulin) is the preferred protein standard. The addition of 1 M NaOH was suggested by Stoscheck (1990) to allow the solubilization of membrane proteins and reduce the protein-to-protein variation in color yield.

Protocol for Bradford assay:

Mix the Bradford ultra reagent solution immediately before use by gently inverting the bottle several times (don't shake the bottle to mix the solution). Remove the amount of reagent needed and equilibrate it to room temperature before use.

Make a dilution series of the chosen model protein in the range: 0,1 mg\ml -1,5 mg\ml (high protein range) OR 1 µg\ml – 25 µg\ml(low protein range)

Mix the samples, standards and a blank (buffer, no protein) with Bradford ultra reagent in microtiter plate:

For 0,1 µg\ml-1,5 µg\ml protein (high range)

20 µg sample – 300 µg Bradford ultra reagent

For 1 µg\ml -25 µg\ml protein (low range)

150 µg sample – 150 µg Bradford ultra reagent

Read absorbance immediately at 595 nm.

Subtract the average 595nm measurement for the Blank replicates from the 595nm measurements of all other individual standard and unknown sample replicates. Prepare a standard curve by plotting the average Blank-corrected 595nm measurement for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

Method:

1. Add to each:

- 1 and 2 test tubes 5µl of bovine serum albumin (0,5mg\ml) and 95µl of NaCl;
- 3 and 4 test tubes 10µl of bovine serum albumin (0,5 mg\ml) and 90µl of NaCl;
- 5 and 6 test tubes15µl of bovine serum albumin (0,5 mg\ml concentration) and 85µl of NaCl;
- 7 and 8 test tubes 100 µl of NaCl;

2.Measure absorbance of each sample on spectrophotometer at 595nm;

3. Prepare standard curve of absorbance versus micrograms protein and determine amounts from the
curve.

4. Graph standard curve (absorbance on the Y axis vs. protein mg/ml on the X axis).

5. Add 8 µl of bovine serum albumin and 92 µl of NaCl ,1 ml of Bradford reagent; measure
absorbance of sample and estimate the protein concentration of the unknown sample by using the slope of standard curve.

Data:

1) 1st and 2 nd test tubes: 5µl of bovine serum albumin (0,5 mg\ml concentration) and 95µl of NaCl A1= 0,126; A2=0,128

$\mu = \Sigma w_i/n$; μ = (0,126+0,128)/2= 0,127

m: 5µl = 0,05 ml

0,5 mg\ml ----1ml

x mg\ml----0,005ml

m= 0,0025 mg=2,5µg

2) 3 rd and 4th test tubes 10µl of bovine serum albumin (0,5 mg\ml concentration) and 90µl of NaCl;

A1= 0,267; A2=0,261

$\mu = \Sigma w_i/n$; μ = (0,267+0,261)/2= 0,264

m: 10µl = 0,01 ml

0,5 mg\ml---- 1ml

x mg\ml---0,001ml

m= 0,0005 mg=5 µg

3) 5th and 6 th test tubes 15µl of bovine serum albumin (0,5 mg\ml concentration) and 85µl of NaCl;

A1= 0,338; A2=0,370

$\mu = \Sigma w_i/n$; μ = (0,338+0,370)/2= 0,354

m: 15µl = 0,015 ml

0,5 mg\ml---- 1ml

x mg\ml----- 0,015ml

m= 0,0075 mg =7,5 µg

4) 7th and 8th test tubes 100 µl of NaCl;

Control samples;

5) 9th test tube

A1= 0,125; A2=0,209

$\mu = \Sigma w_i/n$; μ = (0,125+0,209)/2= 0,167

0,5 mg\ml ---- 1 ml

x mg\ml--0,008ml

m= 0,004 mg=4 µg

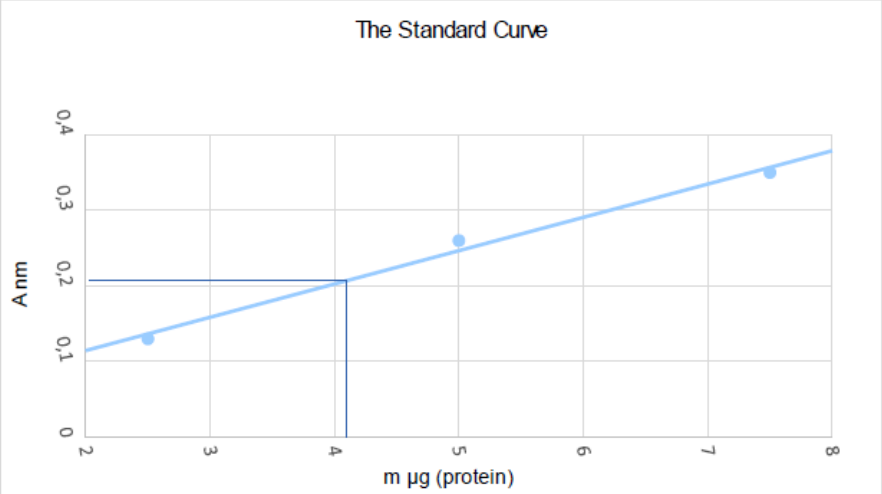

Figure 1 - The standart curve of Bradford assay.

Conclusion

As purpose of this experiment was to find the concentrarion of unknown protein by using standard curve, to fulfill this and to get the standard curve, I used bovine serum albumin (0,5 mg\ml concentration) as known protein. Then, I measured the absorbance of each sample on
spectrophotometer at 595nm. By obtained data I was able to find out the concentration of unknown protein. At the end of this experiment I determined both , theoretical and practical concentration of unknown protein;

Through the experiment, I was able to solve for the concentration of the unknown protein solution by using the linear regression method and by plotting the standard curve by absorbance versus concentration.The absorbance obtained for the unknown was 0.209 nm. The theoretical average concentration was 4 mg and practical average concentration was 4,4 mg (by using the Standard Curve of concentration of BSA.

REFERENCES

1 G. F. Zubay, W. W. Parson, D. E. Vance, Principles of Biochemsitry, WBC Publishers, England, Latest Edition. 2004. - P 13.
2 Harper's Biochemistry, 25th ed New York : McGraw-Hill, Inc., 2002. - P 38.
3 Introductory Practical Biochemistry, New Delhi : Narosa Publishing House, 2002. P 86. Practicals and Viva in Medical Biochemistry, Amsterdam : Elsevier Science. 2004. - P 32.
4 J. Jayaraman, Laboratory Manual in Biochemistry, Willey, Eastern Limited, New Delhi.
5 Koolman, Color Atlas of Biochemistry, 2nd edition © - 2005. - P 100.
6 L. Lehninger, Principles of Biochemsitry, CBS Publishers and Distributors. 2004. - P 32.
7 Lehninger Principles of Biochemistry, 3rd ed London : Macmillan Press Ltd., 2000. - P 32.
8 Lippincott's Illustrated Reviews: Biochemistry Fifth Edition Richard A. Harvey, PhD Professor Emeritus Department of Biochemistry University of Medicine and Dentistry of New Jersey– Robert Wood Johnson Medical School Piscataway, New Jersey Denise R. 2000. - P 246.
1. Ferrier, PhD Professor Drexel University College of Medicine Philadelphia, Pennsylvania
9 M. Cohn, K. S. Roth, Biochemstry and Disease, William and Wilkins Co., Baltiomore, Latest Edition. 2006. - P 246.

А.С.Қожамжарова, Л.С.Қожамжарова,* С.О.Ордабеков, З.Б.Есимсеиитова**
С.Ж.Асфендияров атындағы Қазақ ұлттық медицина университеті
**М.Х.Дулати атындағы Тараз мемлекеттік университеті*
***Әл-Фараби атындағы Қазақ ұлттық университеті*

БИОХИМИЯЛЫҚ ӘДІСТЕР АРҚЫЛЫ ПОЛИПЕПТИДТЕРДІ, АҚУЫЗДАРДЫ ЖӘНЕ АМИНҚЫШҚЫЛДАРЫН САНДЫҚ ЖӘНЕ САПАЛЫҚ СӘЙКЕСТЕНДІРУ

Түйін: Мақалада биуретті талдау туралы ақпарат, бұл әдістің қаншалықты артықшылығы, жылдамдығы, түрлі тиіндермен қалыптас әртүрлі ақуыздармен бірнеше аралас заттардың түстерін тасымалдау сәйкестігі қарастырылған. Оның негізгі кемшілігі сезімталдығының болмауымен ерекшеленеді.
Түйінді сөздер: ақуыздар, биурет, спектрофотометрия, аминқышқылдары, микрограмма, карбон қышқылы.

А.С.Кожамжарова, Л.С.Кожамжарова,* С.О.Ордабеков, З.Б.Есимсеиитова**
Казахский Национальный Медицинский Университет имени С.Д.Асфендиярова
**Таразский Государственный Университет имени М.Х.Дулати*
***Казахский национальный университет имени аль-Фараби*

КАЧЕСТВЕННАЯ И КОЛИЧЕСТВЕННАЯ ИДЕНТИФИКАЦИЯ АМИНОКИСЛОТ, ПОЛИПЕПТИДОВ И БЕЛКОВ ЧЕРЕЗ БИОХИМИЧЕСКИЕ МЕТОДЫ

Резюме: Статья содержит информацию об анализе биурета, этот метод имеет несколько преимуществ, включая скорость, сходную цветопередачу с различными белками и несколько мешающих веществ. Его основным недостатком является отсутствие чувствительности.
Ключевые слова: белки, биурет, спектрофотометрия, аминокислоты, микрограмма, карбоновая кислота.