

EFFICACY OF THE ESTABLISHED METHODS FOR DETERMINATION OF PROTEIN IN FISH VISCERAL EXTRACT

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Abstract : Protein can be quantified by various biochemical assays but all methods are subjected to artifacts. Also, the nature of the sample governs the selection of appropriate method. Fish visceral extract is a complex mixture of bioactive peptides, polypeptides, lipoproteins, hexopeptides etc. Samples containing higher protein concentration vary the results of Bradford assay. On the other hand, Lowry assay and Biuret assay suffer artifacts by the presence of non-proteinaceous components. In this study, protein content in liver and intestinal extracts of *Catla catla* and *Labeo rohita* was determined by the methods of Lowry *et al.* (1951), Gornall (1949) and Bradford (1976). Highest protein content was found in Catla liver by all the methods (1740 mg ml⁻¹, 580 mg ml⁻¹ and 1450 mg ml⁻¹ resp.). Lowest concentration was found in Catla intestine by all the methods (1290 mg ml⁻¹, 200 mg ml⁻¹ and 1030 mg ml⁻¹, resp.). A good association (98.6%) was found among the three methods indicates that these are comparable and can be used for protein quantification in fish visceral extract.

Index Terms - Lowry, Biuret, Bradford, fish visceral waste, chi square test.

I. INTRODUCTION

Fish visceral waste is a mixture of complex substances such as bioactive peptides, polypeptides, lipoproteins, hexopeptides etc. (Murthy *et al.*, 2012). Apart from proteins, fish visceral extract consists of fatty acids (monosaturated acids, oleic acid, and palmitic acid), minerals, and carbohydrates (Ramakrishnan *et al.*, 2013). Toxic substances, at rather low concentration have also been reported (Arvanitoyannis and Kassaveti, 2008). The composition of fish waste makes it applicable as animal feed, manure (Chalamaiah *et al.*, 2012), and source of high valued products such as gelatin, collagen (Herpandi *et al.*, 2011), and high and low molecular weight enzymes (Loganathan *et al.*, 2013). Thus, protein determination in fish visceral extract is essential.

Estimation of protein concentration is necessary in protein purification, electrophoresis, cell biology, molecular biology and other research applications. With the advent of new techniques, the number of assay techniques, each with its own merits and demerits, has also increased. Most protein assays require use of colorimeters or spectrophotometers. Factors considered for selecting suitable assay includes nature and composition of protein (Ng and Cho, 2000), accuracy and sensitivity of the method (Janairo *et al.*, 2011).

The Lowry, Biuret and Bradford methods are the most commonly used dye-binding protein assays. The Lowry assay is based on the principle that under alkaline conditions cupric ions (Cu²⁺) chelate with the peptide bonds of the sample, resulting in reduction of Folin Ciocalteu Reagent (phosphomolybdic/phosphotungstic acid) to heteropolymolybdenum blue, which is read at 650-750nm. The major limitation of the assay is interference due to buffers (at certain level of concentration) commonly used in preparing cell extracts (Peterson, 1979). On the other hand, color transition from blue to violet by biuret reagent occurs due to formation of copper-protein complex between the cupric ions (Cu²⁺) and four nitrogen atoms of peptide chains which is measured at 540nm. The assay is, however, independent of protein composition (Ng and Cho, 2000 and Sapan *et al.*, 1999) but is prone to interference by tris buffer, ammonium ions, sucrose, primary amines, fats and glycerol (Gornall *et al.*, 1991). Thus, the method is comparatively insensitive. Detection of reduced copper using Folin-Ciocalteu reagent makes the Lowry assay nearly 100 times more sensitive than the Biuret reaction (Gornall *et al.*, 1949; Hartee, 1972 and Peterson, 1979).

The Bradford assay is based on direct binding of Coomassie brilliant blue G-250 dye (CBB) specifically to tryptophan, tyrosine, histidine, phenylalanine and primarily to arginine residue present in protein sample (and not to small peptides and amino acids) (Kamizake *et al.*, 2003). The assay is free from phenolic interference and has gained popularity because of high sensitivity, perceived linearity, speed and ease of performance (Redmille-Gordon *et al.*, 2013). Although interference by chemicals such as glycerol, detergents, 2-mercaptoethanol, acetic acid, ammonium sulfate, tris, and alkaline buffers can be corrected using suitable standard, but the concentration of interfering agents need to be known for the same (Spector, 1978). However, the unstable reaction color (6-8% change/hr) as compared to 1-2% change/hr in Lowry (Berden, 1978) has restricted its wide use.

In the present study, the protein content of *Catla* and *Labeo* visceral extract was determined by Lowry, Bradford and Biuret assays and their dependency over protein sample was checked.

II. RESEARCH METHODOLOGY

Fish sample

The liver and intestine of *Catla catla* and *Labeo rohita* were procured from local fish market. Samples were kept in ice and immediately brought to laboratory and stored at -20 °C in deep freezer (Blue Star).

Preparation of crude enzyme extract

Liver and intestine of both fishes were washed with cold water (20-24°C). The washed viscera were homogenized separately in extraction buffer (containing 1mM Tris-HCl; 10mM CaCl₂; pH 8) at 4°C. The homogenate was then centrifuged at 10,000g; for 30 min at 4 ° C using Eppendorf Centrifuge (5415R). The supernatant (enzyme extract) was collected and stored at -20° C (Simpson and Haard, 1984).

Protein determination

BSA (HiMedia, RM3155-100G) was used as standard (0-2000 mg/ml concentration) for all the three assays in which crude enzyme extract served as sample. The chemicals of Lowry method, Bradford method and Biuret method were purchased from HiMedia. The protein content was determined by UV/VIS spectrophotometer (DU 730 Beckman-Coulter).

Statistical analysis

Data was analyzed by Chi-square test to assess dependency of protein assays upon sample type. The assays were hypothesized to be independent of the sample. Finally, the Coefficient of Association (Φ) was calculated using the formula-

$$\phi = \frac{\sqrt{\chi^2 * 100}}{\sqrt{\chi^2 + n}}$$

Where, Φ = coefficient of association

χ^2 = chi-square value

n = degree of freedom

III. RESULTS AND DISCUSSIONS

Protein determination

Fig 1 shows highest protein content in rohu liver and lowest in rohu intestine. Of the three methods, lowest values were obtained by Biuret method, followed by Bradford and Lowry. Biuret method has been reported as the least sensitive assay lowering the protein value (Martina and Vojtech, 2015). Presence of low molecular weight substances lower the concentration value more in Biuret method than in Bradford and Lowry's method (Williams *et al.*, 1995). While measuring protein in *Aspergillus niger*, Jernejec *et al.* (1986) also obtained lower protein value with Bradford method as compared to Lowry's method. The dependency of Bradford method upon sample containing a mixture of proteins has been emphasized by Ng and Cho, (2000). Consistent values have been obtained with Lowry's method as compared to Bradford method. Lu *et al.* (2010) have emphasized over higher sensitivity of Lowry's method as a reason for higher protein value while quantifying and identifying protein expression by Lowry and Bradford methods. Higher sensitivity of Lowry's method has also been suggested by Malin and Ridzuan (2010), Anggun (2013) and Martina and Vojtech (2015). The liver sample exhibited more protein than intestine. Also, visceral protein in Rohu was more as compared to that in Catla. The results were in accordance with the results of Kumar (2014).

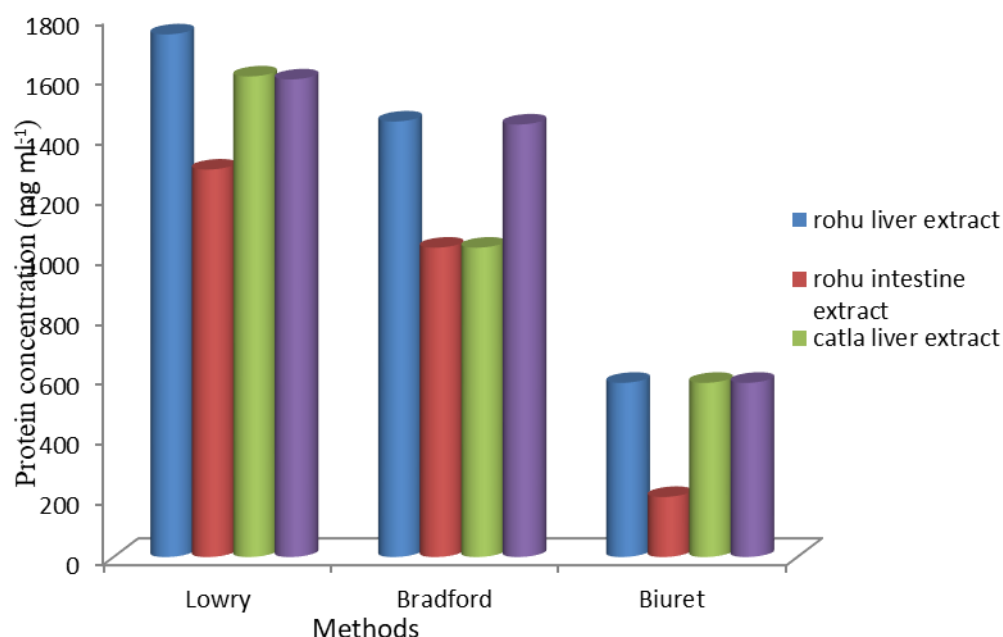


Fig.1 Protein comparison as determined by Lowry, Biuret and Bradford assays in fish visceral extracts

Statistical analysis

Using the values of protein concentration, contingency Table was constructed as Table 1. Based on chi-square test, the results were significant (p value ≤ 0.05 with 6 degree of freedom) and the coefficient of association was found to be 98.6% which is indicative of a very good association.

Table 1 Contingency table

	Rohu Liver	Rohu Intestine	Catla Liver	Catla Intestine	Total
Lowry	1740	1290	1600	1590	6220 (R1)
Bradford	1450	1030	1030	1440	4950 (R2)
Biuret	580	200	580	580	1940 (R3)
Total	3770 (C1)	2520 (C2)	3210 (C3)	3610 (C4)	13110

IV. CONCLUSION

Of the three spectroscopic methods to determine protein content, Lowry's method gave the highest values followed by Bradford and Biuret method. The complex nature of visceral extract was found to affect the results. However, a good association (98.6%) existed among the three methods, Lowry's method has been suggested to be most appropriate as it is unaffected by the sample nature.

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