Analysis of Protein by Spectrophotometric and Computer Colour Based Intensity Method from Stem of Pea (*Pisum sativum*) at Different Stages

Afsheen Mushtaque Shah*[1], Mohammad Saleh Memon[1], Allah Nawaz Memon[1], Abdul Wahab Ansari[2] and Basir Ahmed Arain[3]

[1]Institute of Biochemistry, University of Sindh, Jamshoro, Pakistan
[2]Department of Computer Science, ISRA, University, Hyderabad, Sindh, Pakistan
[3]Institute of Plant Sciences, University of Sindh, Jamshoro, Pakistan

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Abstract

In this study proteins were analyzed from pea plants at three different growth stages of stem by spectrophotometric i.e Lowry and Bradford quantitative methods and computer colour intensity based method. Though Spectrophotometric methods are regarded as classical methods, we report an alternate computer based method which gave comparable results. Computer software was developed the for protein analysis which is easier, time and money saving method as compared to the classical methods.

Keywords: Protein, Spectrophotometer, Computer software, Dye and colour intensity.

Introduction

Pea, the botanical name of which is *Pisum sativam*, belongs to family fabaceae. It is an important vegetable used in diet and a good source of protein. Protein is important nitrogenous macromolecules that are composed of amino acids linked together by peptide linkages [1]. Proteins play significant biological function in human as well in plants. These can be analyzed from seeds and other parts of plants such as leaves and stems [2-4]. Protein synthesis occurs in leaves and green stems and is mobilized into seeds or fruits [5]. Proteins have been assayed from almost all parts of plants including leaves, nodules, stem, fruit and seed [6, 7].

A number of dyes have been used in dyeing of proteins for example Lawsone and Coomassies dyes. Lawsone is used to dye hair, nail and skin proteins, albumin, casein, a milk protein [8] and staining of finger proteins for finger prints [9].

Proteins have been extracted, identified and analyzed by using different analytical techniques. At present, extra cellular and intra cellular proteins have been isolated and quantitatively estimated by spectrophometrically [10-12]. A number of studies have been conducted in which proteins are analyzed by staining of anatomical sections at desired areas using different dyes [13] like Lawsone and Coomassie Brilliant Blue Dye [14] and the colour intensity measured by computer software. This type of quantitative image processing is extensively used in biological specimens, in diagnosis of disease [16], and in drug analysis [17].

In present study, we have developed computer software, referred as ABAS, for quantitative determination of proteins using lawsone dye in three different stages during pea stem growth and then comparing the results obtained with other spectrophotometeric results.

*Corresponding Author Email: asheeshah@hotmail.com

Experimental
Plantation of pea

Pea was sown in first week of December 2008 by drilling method on the experimental field of Institute of Plant Sciences, University of Sindh, Jamshoro. Plot size for each crop was 10 x 10 meters and normal agronomic practices were performed from sowing till harvest. Stem samples were collected at three different growth stages, that is, after 2 months (pre-flowering Stage), after 3 months (flowering Stage) and after 4 months (fruiting Stage) denoted P-1, P-2 & P-3 for each the stages of pea. At each growth stage stem samples were collected from lower, middle and upper part of the same plant and replicated four times.

Determination of proteins by spectrophotometric methods
Preparation of reagent/chemicals

Phosphate Buffer (0.1 M, pH-7.6) was prepared by mixing 16.0 ml of (i) and 84.0 ml of (ii) and adjust the volume to 200 with distilled water. A 0.2 M monobasic sodium phosphate by taking 27.8 g of NaH_2PO_4 in a volumetric flask and the volume adjusted to 1 liter with distilled water (i). A 0.2 M dibasic sodium phosphate Na_2HPO_4. $7H_2O$ by taking 53.6 g of Na_2HPO_4 volumetric flask and the volume adjusted to 1 liter with distilled water (ii). Alkaline Na_2CO_3 reagent was prepared by taking 2.0 g of Na_2CO_3 and dissolved in 0.1 N NaOH and volume adjusted to 100 ml. Take 0.5 g of $CuSO_4.5H_2O$ and add 1.0% sodium potassium tartarate and adjust the volume upto 100 ml to prepare copper sulfate reagent. The copper sulfate was made alkaline by mixing 1.0 ml of copper sulfate reagent in 50 ml of alkaline sodium carbonate reagent. Bovine Serum Albumin (BSA) was diluted by taking 100 µg of bovine serum albumin and distilled water is added to make the volume to 100 ml.

A 100 ml of Coomassie Brilliant Blue G-250 was dissolved in ethanol and add 100 ml of 85% of phosphoric acid and make the volume up to 1liter with distilled water (Bradford Reagent).

Extraction of proteins

1.0gram of each plant position (P-I, P-2 and P-3) was ground in pastel mortar with 1.0 ml of phosphate buffer. These samples were kept overnight for complete extraction of protein. These were centrifuged at 16. 163g for 20 minutes. The supernatant is used for protein analysis and the pellet is discarded [18].

Procedure for lowry's method

To 1.0 ml of supernatant from above is added 5.0 ml of alkaline copper sulfate reagent and thoroughly mixed. Allowed to stand for ten minutes and then add 0.5 ml of Folin's reagent. In order to develop colour this is kept standing for 30 minutes. This was followed by recording absorbance in spectrophotometer at 660 nm, against a blank. The blank is prepared by taking 1.0mL of 0.5 M NaOH in place of sample in cuvette. Bovine serum albumin is used to draw a standard curve and the amount of proteins in different samples are estimated [8, 18].

Procedure for bradford method

Take 0.5 ml of supernatant and adjust volume to 1.0 ml with phosphate buffer and to this 50.mL of Bradford reagent was added and absorbance recorded at 595 nm against reagent blank. A standard calibration curve is drawn by using bovine serum albumin as standard [9, 10 & 18]. From the standard curve, the concentrations of proteins in samples were obtained.

Computer colour intensity based method
Preparation of sections

Cross sections of different stages of pea Stem (P-1, P-2 and P-3) are prepared by cutting it with razor blade-Trig [19].

Following dyes are prepared for staining.
1. Coomassie Brilliant Blue G-250 (CBB dye) [12].
2. Lawsone dye
   a) Pure Lawsone dye (Ls dye).
   b) Lawsone dye: Extracted from Henna powdered leaves for one hour (Hple1).

Preparation of coomassie brilliant blue G-250 (CBB dye)

The dye is prepared by taking 25 mg of CBB-G-250 in 12.5 ml of pure ethanol and 25 ml of 85% phosphoric acid and then making the total volume upto 250 with distilled water. To prepare de-staining solution an equal amount of methanol and 7% acetic acid (1:1 ratio) is taken.

Preparation of pure lawsone dye (Ls dye)

To 2 gram of lawsone dye distilled water was added and the volume adjusted to 100 ml (2% pure lawsone) [20].

Preparation of lawsone from Henna (Lawsonia inermis) powdered leaves (Hple1)

Henna leave are taken and completely dried in shadow, followed by grinding in grinder to get henna in powdered form for analysis. To 2 gram of powdered henna 100 ml of distilled water is added and let it stand for 60 minutes. This is then filtered and the extract is used for staining of different plant sections. These were de-stained with distilled water.

Determination of protein by computer software
Preparation of sections for microscope

Anatomical sections of three different stages of pea stem were prepared for analysis. Before staining each section was placed on light microscope (XSN 107 BN, China) [21]. The microscope was then connected to computer through digital camera (ETUSB-2710), using UBS port. 25×magnification is used for all observations. The cross sections [2D image] appear on computer monitor. These are saved in stained and unstained forms.

Staining of C.S of pea (P-1, P-2 & P-3) with CBB dye

Cross sections of pea's stem (P-1, P-2 and P-3) are placed in CBB stains for 1 hr, 2 hrs, 3 hrs and 24 hrs at room temperature. These sections are observed under microscope and 2D images are captured and saved in computer memory for protein analysis. These sections are de-stained for 30 minutes and observed under microscope and their 2D images are captured and saved in computer memory for protein analysis.

Staining of pea sections with Ls dye

Cross sections of Pea stem of all three stages (P-1, P-2 and P-3) stained in 2% Lawsone dye (pH-4) for 1hr, 2hrs, 3hrs and 24hrs at room temperature. Each time section was destained for 30min. Before and after staining (unstained and stained), sectioned were observed under microscope and image was saved in computer hard disk for analysis.

Staining of C.S of pea (P-1, P-2 & P-3) with Hple1

Cross section of stem of Pea (P-1, P-2 & P-3) stained in 2% Hple1 (pH-4) for 1hr, 2hrs, 3 hrs and 24 hrs at room temperature and de-stained for 30 min. Before and after staining section were observed under microscope and images were saved in computer memory for protein analysis by computer software.

Determination of colour intensity of 2D images (plant sections)

ABAS computer software was specially developed for the quantification of colour intensity of 2D images. This software recognizes up to 0.1 mg (weight of section) sections. So after cutting series of sections, software will only select section (0.1 mg weight) due to constant intensity of its 2Dimage. This is an advantage of this software. There will be no need of Microtome for plant sections. First of all sections average Background of Optical Density (BOD), which is screen of window without plant section was calculated and then calculate the Optical Density of section which is the sum of Red, Green and Blue colour intensities. 554.25 ou/μm^2, it is constant value which is sum of Optical Density of Red, Green, Blue and Black colour.

Following equation was used to calculate total intensity (TI) of colour.

$$IT_{2Dimge} = 554.25 \frac{554.25 \times \sum OD\,ou/\mu m^2}{\sum BOD} \quad [22, 23].$$

After calculation of Total intensity (TI) of each section, TI of Stained image was subtracted from the TI of Unstained image that will be called as Total intensity of Dye (TID) only.

TID = TI of Unstained Image – TI of Stained Image.

The value of TID shows the amount of protein, because Lawsone dye is specific dye for Protein hence TID divided by TI of weighted image (0.1mg), which is fixed in Software.

$$\% \text{ of Protein} = \frac{\text{TID}}{\text{TI of weighted image}} \times 100$$

Results
Protein from three stages of pea (P-1, P-2 & P-3) by spectrophotometric methods

Total protein contents are determined by spectrophotometric method and then assessed by computer software. P-1 shows 1.24 mg/100 g protein by Lowry method and 1.68 mg/100 g by Bradford method respectively. P-2 shows 4.55mg/100 g by Lowry and 4.63 mg/100 g by Bradford method, whereas in P-3, the values of proteins are 4.55 mg/100 g by Lowry and 4.63 mg/100 g by Bradford method respectively. Our results show that the content of proteins is slightly increased in P-1 and P-2 by Bradford method as compared to Lowry method. However the protein content found by Lowry's methods is more in p-3 as compared to Bradford method. (Fig. 1 and Fig. 2).
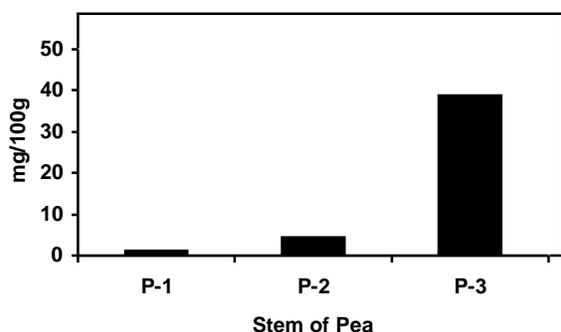

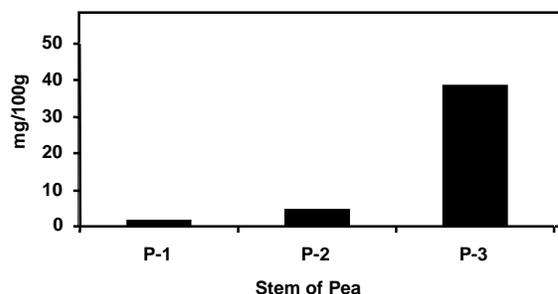*Figure 1.* **Stem of pea: Protein analysis by Lowry method from three stages.**

Figure 2. **Stem of Pea: Protein analysis by bradford method from three stages.**

Staining of anatomical sections with CBB dye

Coomassie Brilliant Blue (CBB dye) is widely used in electrophoresis techniques for the staining of Protein [12]. In this research work, first of all Coomassie dye was used in Bradford method then it was used for the staining of Cross sections of stem of Pea (P-1, P-2 & P-3). Though CBB dye was successfully stained the anatomical sections of plants but computer software computed the colour intensities of stained plant sections and the colour of CBB dye is dark blue colour, that is why it gave highest concentration of protein contents than Ls dye & Hple1 and also Spectrophotometric methods, this study proved that light colour staining dyes should be used for the staining of anatomical sections of the computer software assessment.

Staining of anatomical sections with lawson dye

Lawsone dye is a natural dye which is extracted from leaves of Henna. Lawsone dye stained the skin, hair and nail Protein [20] and same dye was used by Ali R *et al* 1988 [8] for the staining of Protein in Electrophoresis technique and now it is used for staining fingerprints reported by Jelly *et al* 2008 [9], in this study first of all this dye was used in Paper Chromatography for the staining of Protein after that same dye was used for the staining of Protein through the anatomical sections of Plants. Pure Lawsone dye is expensive, we can extracted this dye from the Henna leaves as reported by Ali R *et al.* 1988 [13] but the quality of dye is less or during extraction process dye molecule may destroy or demise. And Henna paste or Powdered leaves extraction are freely used for the staining of Protein of hair, skin and nails, for

the economical purpose of this method, first of all plant's sections were stained with pure Lawsone dye (Ls) and then stained with Henna powdered leaves extraction for one hour (Hple1). Hple1 gave less protein quantity then Ls dye solution by computer method through anatomical sections of plants.

ABAS computer software assessment of three growth stages of aea:

P-1 the first stage of pea plant development/ growth

The computer method used for estimation of protein content in different sections of pea, stained with different dyes i-e CBB dye, Ls dye and Hple1 at deferent time intervals, show variation in protein content. (Fig. 3) shows concentration of proteins mg/100 g in section P-1 appeared maximum after 2hrs staining with CBB dye (3. 69 mg/100 g), with Ls dye it is 1.88 mg/100 g (Fig. 4) and 1.71 mg/100 g with Hple1 (Fig. 5).
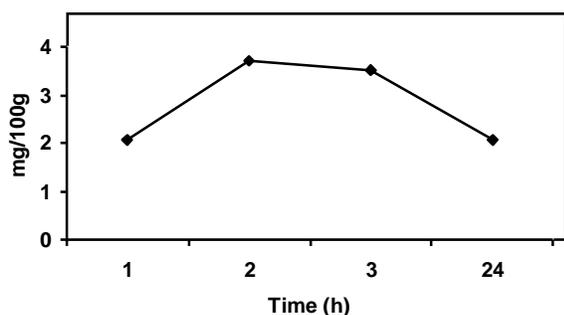
Figure 3. **Stem of Pea (P-1): C.S stained with CBB dye for the assessment of protein for different time intervals at room temperature.**
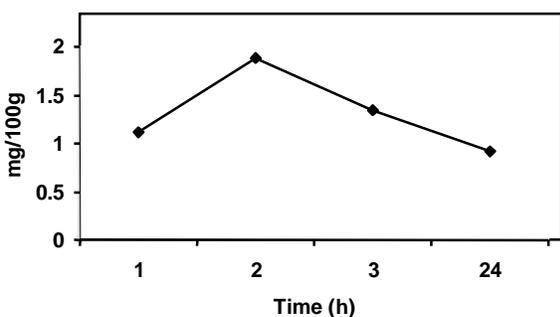

Figure 4. **Stem of Pea (P-1): C.S stained with Ls dye for the assessment of protein for different time intervals at room temperature.**
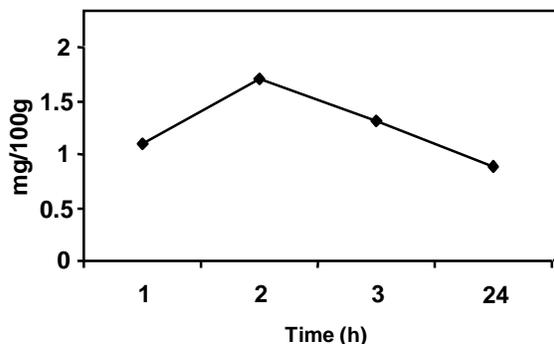

Figure 5. **Stem of Pea (P-1): C.S stained with Hple1 for the assessment of protein for different time intervals at room temperature.**

(Fig.6) shows that protein content in cross section of stem of pea (P-1) stained with CBB bye is increase as compared to those stained with Ls dye and HPle1 at different time interval and the protein content is lower in the P-1 stained with Hple1 (Fig. 6). The mean value of conch of protein is significantly high (2.8 ±0.44 mg/100 g) in sample stained with CBB dye as compared to mean value of 1.31 ± 0.21 mg/100 g with 1-g dye and 1.25 ± 0.7 mg/100 g with Hple1 dye respectively (Fig. 7) . The result reveals significance difference ($p < 0.01$) in conch of protein stained with CBB dye as compared to that of 1-g and HPle1 stained samples. However, the results are not significant between 1-g and HPle1 stained samples.
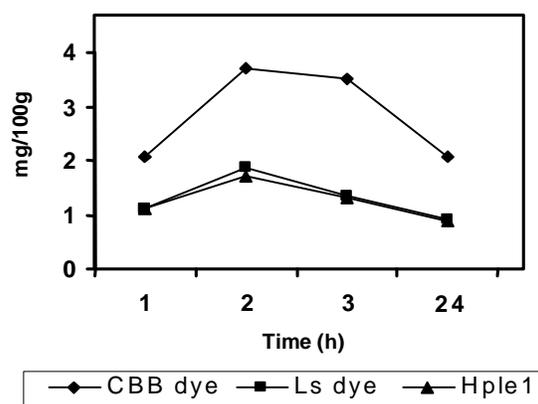

Figure 6. **Comparison of staining of C.S of Pea (P-1) with CBB dye, Ls dye & Hple1 for the assessment of protein for different time intervals at room temperature.**
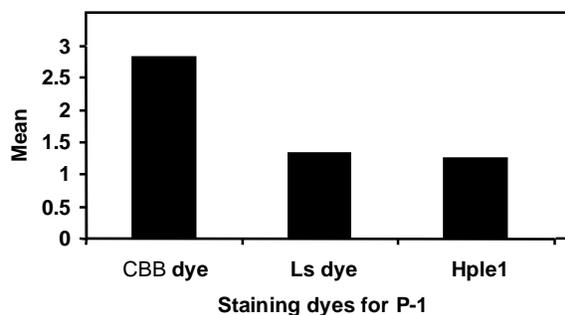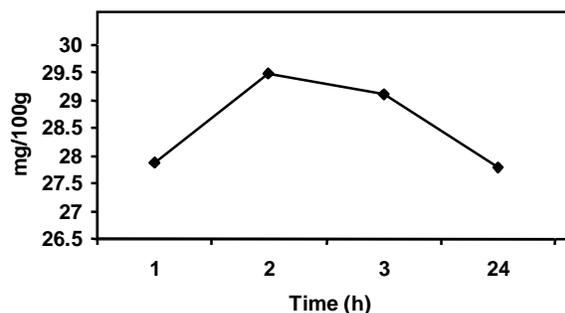
Figure 7. **Comparison of mean between Pea (P-1): C.S stained with CBB dye, Ls dye & Hple1 for the assessment of protein for different time intervals at room temperature.**

P-2 the first stage of pea plant development/ growth

The protein concentration assessed by computer software incase of P-2, stained sections with three different dyes is shown in (Fig. 8-11) Figures shows the cumulative/ comparative results of protein concentration at different times intervals of 1hr, 2hrs, 3hrs and 24hrs.

Figure 8. **Stem of Pea (P-2): C.S stained with CBB dye for the assessment of protein for different time intervals at room temperature.**
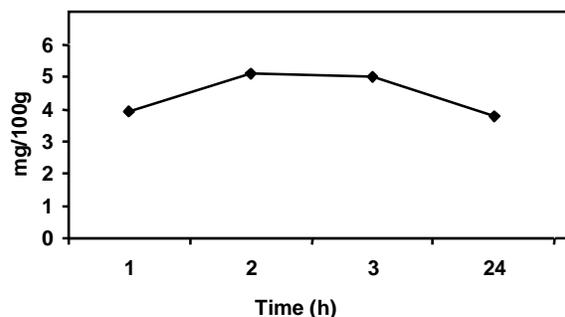

Figure 9. **Stem of Pea (P-2): C.S stained with Ls dye for the assessment of protein for different time intervals at room temperature.**
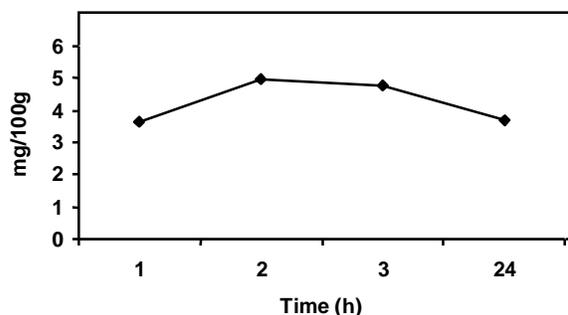

Figure 10. **Stem of Pea (P-2): C.S stained with Hple1 for the assessment of protein for different time intervals at room temperature.**
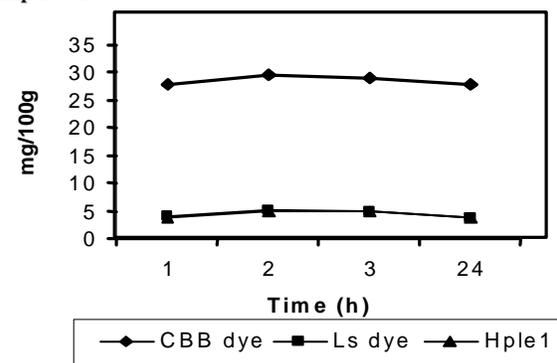

Figure 11. **Comparison of staining of C.S of Pea (P-2) with CBB dye, Ls dye & Hple1 for the assessment of protein for different time intervals at room temperature.**

All the graphs show maximum concentration of proximate at 2hrs intervals. 1hr and 24hrs intervals showed approximately same results. 3hrs intervals were between the 1hr, 24hrs and 2hrs intervals. Temperature was kept constant at room temperature.

Fig. 24 show the concentration of protein in the sections stained with CBB dye at 1hr, 2hrs, 3hrs and 24hrs intervals was 27.88/100g, 29.48mg/100g, 29.11mg/100g and 27.8mg/100g of plant stem.

When sections stained with Ls dye, (Fig. 9) shows the protein concentration was found to be 3.91mg/100g, 5.12mg/100g, 5.0mg/100 and 3.78mg/100g of plant stem in case staining for 1hr, 2hrs, 3hrs and 24hrs respectively. In this case same pattern of concentration has been followed as in case of CBB dye.
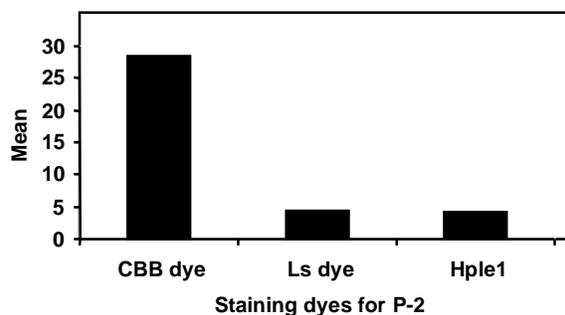
Figure 12. **Comparison of mean between Pea (P-2): C.S stained with CBB dye, Ls dye & Hple1 for the assessment of protein for different time intervals at room temperature.**

P-3 the first stage of pea plant development/ growth

Section stained with CBB dye, (Fig. 13) shows the concentration of protein at third stage of plant stem. The sections were dyed for 1hr, 2hrs, 3hrs and 24hrs and the protein concentration was found to be 30.33mg/100g, 45.55mg/100g, 45.01mg/100g and 42.55mg/100g of plant stem. The concentration in this assessment is not very high as compared to previous P-1 and P-2 stages. The readings are very near to other dyes. The pattern of concentration of protein at different intervals is also same i.e 2hrs. Staining period shows maximum concentration while 1hr, and 24 hrs staining shows minimum and 3hrs staining give the concentration in between 1hr, 24hrs and 2hrs staining periods.
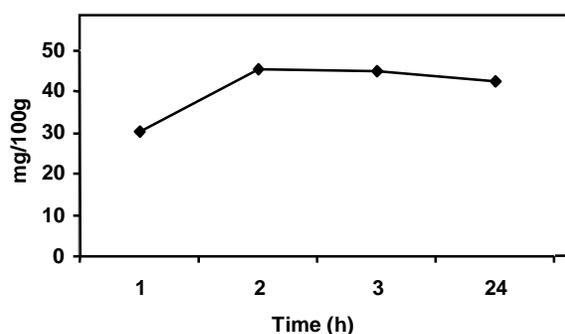

Figure 13. **Stem of Pea (P-3): C.S stained with CBB dye for the assessment of protein for different time intervals at room temperature.**

Staining with Hple1 dye: (Fig. 10) shows the content of protein in anatomical sections stained with third dye, in these sections the concentration has been show as 3.66mg/100g, 4.96mg/100g, 4.75mg/100g and 3.69mg/100g of plant, when staining of the sections was carried out for 1hr, 2hrs, 3hrs and 24hrs. Again the same pattern of concentration has been following in this case also.

Comparative graph for all the three dyes in (Fig. 11) comparative results has been produced. The fig shows that the concentration of protein in the stained dye with CBB dye show a very large difference while two other dyes Ls and Hple1 are having the vary close concentration. Comparative of mean concentration of protein of sections stained with three different dyes and at different time intervals at room temperature. In (Fig.12) Mean values shows that maximum concentration is with CBB dyes while both Ls and Hple1 shows same concentration.

Staining of P-3 with Ls dye: (Fig.14) gives as the concentration of protein in the sections stained with Ls dye. After 1hr, 2hrs, 3hrs and 24hrs staining, the concentration of protein is shown to be 29.61mg/100g, 40.10mg/100g, 39.41mg/100g and 37.10mg/100g of plant stem material respectively. Again two hours staining gave maximum while other staining period follow the same pattern as in P-1 and P-2 and CBB dye with P-3.
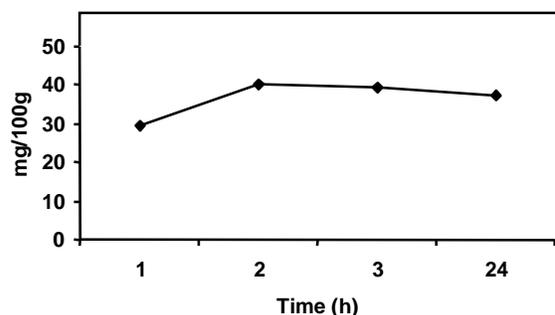

Figure 14. **Stem of Pea (P-3): C.S stained with Ls dye for the assessment of protein for different time intervals at room temperature.**

Staining of P-3 sections with Hple1 dye: In (Fig. 15) it is shown that in case 2hrs staining also give maximum concentration i.e 39.81mg/100g of plant stem. The 3hrs staining show not more amount than 2hrs staining period and that is 38.11mg/100g. A 1hr and 24hrs staining gives 27.44 and 36.8mg/100g, respectively.
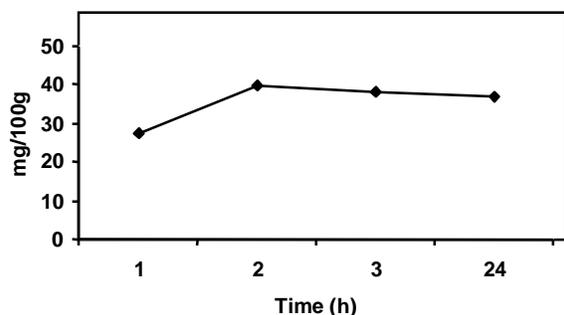
Figure 15. **Pea (P-3): C.S stained with Hple1 for the assessment of protein for different time intervals at room temperature.**

Comparative results of protein concentration with three different dyes; in (Fig. 16) in case of P-3 the results of CBB dye staining is slightly high as compared to both the other dyes. The (Fig. 17) shows comparison of Mean of protein concentration at CBB dye shows high concentration of protein then other two staining dyes.
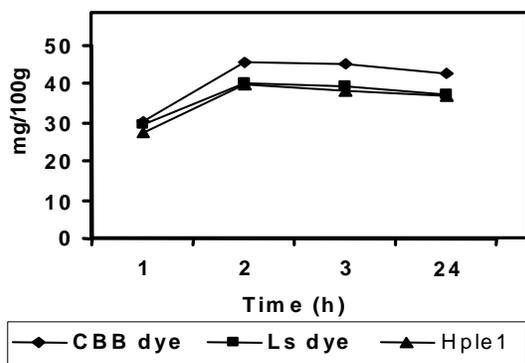

Figure 16. **Comparison of staining of C.S of Pea (P-3) with CBB dye, Ls dye & Hple1 for the assessment of protein for different time intervals at room temperature.**
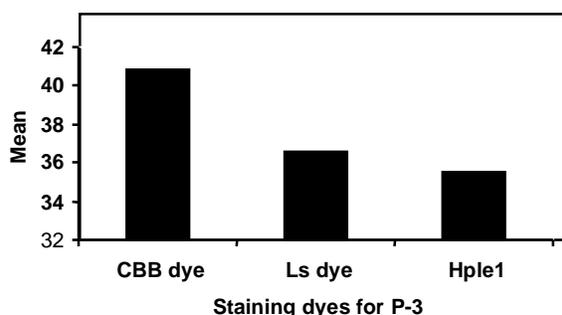

Figure 17. **Comparison of mean between Pea (P-3), C.S stained with CBB dye Ls dye & Hple1 for the assessment of protein for different time intervals at room temperature.**

Discussion

In order to differentiate between different parts of the same plant or between other plants and see the structure and function in relationship anatomical study is necessary. Anatomical sections are important in getting different and more information about the plant structure. This can only be obtained through microscope [21]. The combination of microscope and computer has been applied in many research works. Use computer is not only for saving time or money but it is used for accurate and reliable measurement of different compounds in biological samples. These computer tools are widely used in the medical science especially for diagnosis purpose [24-27]. The present research educates the use of computer in the field of research. The present work shows the comparison of Spectrophotometric methods and computer software amount of protein concentration at different stages of plant development. This technique is another version has also been used by De Mattos *et al* 2006 [22]. He used it for the assessment of immunochemistry of samples by manual and computer software. The anatomical sections were stained with CBB dye, Ls dye and Hple1 for determination of protein through computer software development by the author. The data so obtained was compared with results assessed by Spectrophotometric methods. The results shows that the Lawsone dye extracted (Hple1) or pure form (Ls) have shown same concentration by the computer software and Spectrophotometric determination. The CBB dye in case of P-1 and P-2 shows in great difference while in P-3 the results are close for all the three dyes. The high concentration of protein with CBB dye may be attributed to the colour of the dye which has more intensity as compared to light orange colour of Lawsone dye. This gives as the idea that for computer based studies use light dyes is preferable. The concentration of protein at different stages of development has been assessed it is found that the increase in concentration is due to plant growth. During the plant growth the concentration is increased as has been shown by Shiltz et al 2005 and other authors [3, 5, 6]. Hence P-3 stage showed maximum amount of proteins. The staining period of dye is also established. The staining of sections with all three dyes for 2hrs

gave maximum amount of protein as compared to 1, 3 and 24hrs of staining periods.

Conclusion

Aim of this study was to develop a novel, economical and simple method for the protein analysis. First of all protein was assessed by spectrophotometric then by computer software method. Spectrophotometric results shows that as the plant grew, quantity of protein also increased, same results were observed in computer assessment method. This study proved that computer method can be used for the quantification of proteins through anatomical sections of plants. By this research it is also proved that Lawsone dye can used for the staining of protein through anatomical sections of plants.

Acknowledgment

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