Optimization of assay for total protein in the haemolymph of the honeybee (*Apis mellifera* L.)

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Abstract: Protein levels in honeybee is a credible marker of a bee physiological status. Bradford's method is suitable for quantification of the total protein in the haemolymph. Two types of diluents, different diluting ratios and impact of storage were compared. Average decrease of the total protein content after one week storage of samples was lower in samples diluted in the phosphate buffer pH 7 in comparison with ones diluted with MilliQ water. The haemolymph samples dissolved in the phosphate buffer showed significantly higher yield and lower variability of the total protein content compared to the variability obtained by dissolving the haemolymph in MilliQ water. These results indicate that 200× dilution in the phosphate buffer (pH = 7) seems to be optimized for determination of the total protein in the bee haemolymph.

Key Words: haemolymph, *Apis mellifera*, total protein, Bradford's method, low volume sample

INTRODUCTION

Many proteins circulating in the haemolymph are created in the fat body (Lensky and Rakover 1983). The highest rate of protein synthesis in workers corresponds with the highest protein levels in the haemolymph (Crailsheim 1990). Quantity of the haemolymph protein is dependent on many factors: physiological status of a worker bee: "hive/forager" or "summer/winter" (Fluri et al. 1982), nutrition (Bitondi and Simões 1996, Cremonz et al. 1998, De Jong et al. 2009) and the hygienic abilities of the colony (Lazarov and Zhelyazkova 2018).

Inappropriate protein availability reduces bee longevity, brood rearing and honey production (Crailsheim 1990). Therefore, the total protein in the haemolymph is an important physiological parameter in the honeybee. The quantification protein for extra low volume samples was proposed by Bradford (1976). This spectrophotometric assay is very reproducible and rapid based on the dye binding process. The method is generally well evaluated and currently recommended as the protocol also for the bee haemolymph (Hartfelder et al. 2013).

The object of this contribution is optimization of the Bradford's method for our lab conditions due to our concurrent experiments on the winter bees. We compared different dilution and types of diluents for the haemolymph assay and also try to find the impact of type of storage on repeatability of measurements, i.e. very likely the stability of protein in the diluent until the test performing.

MATERIAL AND METHODS

Chemicals

Phosphate buffer (pH = 7) was prepared by mixing di-Sodium hydrogen phosphate anhydrous (11.876 g/l) and Sodium phosphate monobasic monohydrate (9.078 g/l). MilliQ water (demineralized water) was produced on a Milli Q RG (Millipore, Mass., USA). All chemicals were purchased from Sigma-Aldrich (St. Louis, USA).

Haemolymph collection

Workers were sampled on brood of the same colony and after that kept for a few minutes at 4 °C to immobilize them. The haemolymph sample from each worker was collected in volume 1 µl with using micro-capillary pipette by incision between 3rd and 4th abdominal tergites. The collected haemolymph was put into 1.5 ml Eppendorf tube with addition of tested diluents (MilliQ water of phosphate buffer pH7) with dilution 20×, 30×, 40×, 50×, 100×, 200×, 300×, 400× and 500×. Thus we obtained 2×9 samples from 18 workers applicated for presentation of results in Figures 1–3.

There were collected also paired samples for comparison results depended on applicated diluent in optimized dilution (n = 6, Figure 4). The haemolymph from the same bee in volume 3 µl was put into 0.5 ml Eppendorf and stirred up. Immediately after, always the 1 µl of the haemolymph was 200× diluted in the MilliQ water and the phosphate buffer (pH = 7) and analysed. Thus, six bees were paired-sampled.

Total protein quantification by method Bradford's assay

Bovine serum albumin (BSA) was used as a standard and was used for the validation of the method. Calibration curve was measured within the range from 0.50 to 0.03 mg/ml. Validation of the method using the BSA standard always took place before each determination (the standard was always prepared fresh). Ready-to-use protein reagent Dry Reagent Concentration (BIO-RAD, California, USA) was purchased. This reagent was diluted 1:4 with MilliQ water before analysis (the reagent was always prepared fresh). Determination procedure: 10 µl of the sample was pipetted into a 96 well microtiter plate Nunc Immuno (Fisher Scientific, Pardubice, Czech Republic). Subsequently, 200 µl of diluted protein reagent was added to 10 µl of the sample. Followed by incubation for 5 min at room temperature and absorbance measurement at 595 nm using instrument Infinite M200Pro (Tecan, Männedorf, Switzerland). Samples were measured immediately after the haemolymph collection and eventually some of them again after the one week storage in the freezer (-18 °C). Each sample has been analysed three-times (triplet).

Statistical evaluation

Values in graphs represent mean ± standard deviations (SD). Statistical significance of differences between average values were analysed with the Student's t-test. Values of the total protein in fresh and stored samples of haemolymph were compared to find the minimal differences (Figure 3 red oval). Statistical significance of differences in the paired samples were tested by paired t-test. The accuracy of the assay was estimated from average coefficient of variation (v_x) (Figure 4B).

RESULTS AND DISCUSSION

The results obtained in presented study are from two experiments i) influence of dilution and ii) testing of stability in storage in freezer.

The reason application of the dilutions is that haemolymph is very complex sample which may result in masking the real concentration of determined analyte. Therefore it is important to find the optimal dilution ratio which is in the same time in the linear dynamic range of the calibration curve of the assay method. The total protein in samples measured immediately after the collection of haemolymph with the phosphate buffer pH = 7 was higher than with MilliQ water (Figure 1 and 2).

Differences in the total protein content on dependence of used diluent was noted after the 1 week storage of samples in the freezer (-18 °C) (Figure 1A, 2A). The values of the total protein content in samples diluted with MilliQ water differed after 1 week significantly (p = 0.003) and decreased in average 5.7 µg/µl unlike the samples diluted in the phosphate buffer where the decrease was insignificant (p = 0.665) and only 1.9 µg/µl. The decrease values of the total protein after 1 week

of storage differed significantly in dependence of the used diluent (p = 0.0002). The BSA calibration curve in phosphate buffer (Figure 2B) provides a better standard response due to a more stable reaction with Bradford's reagent than the BSA calibration curve in water (Figure 1B). This phenomenon can be caused by the buffer provides free ions in higher concentrations, and these can better stabilise the counter ions in the structure of protein by surrounding them. This prevents any change in secondary and ternary structure of bigger proteins. This statement was confirmed by other results, where samples of haemolymph dissolved in phosphate buffer were much more stable than samples of haemolymph dissolved in water.

*Figure 1 (**A**) Total protein content in the samples of a bee haemolymph diluted 20–500× in MilliQ water (determination immediately after the collection and after the one week storage in the freezer). (**B**) The calibration curve of BSA in MilliQ water in range 0.50 to 0.03 mg/ml.*

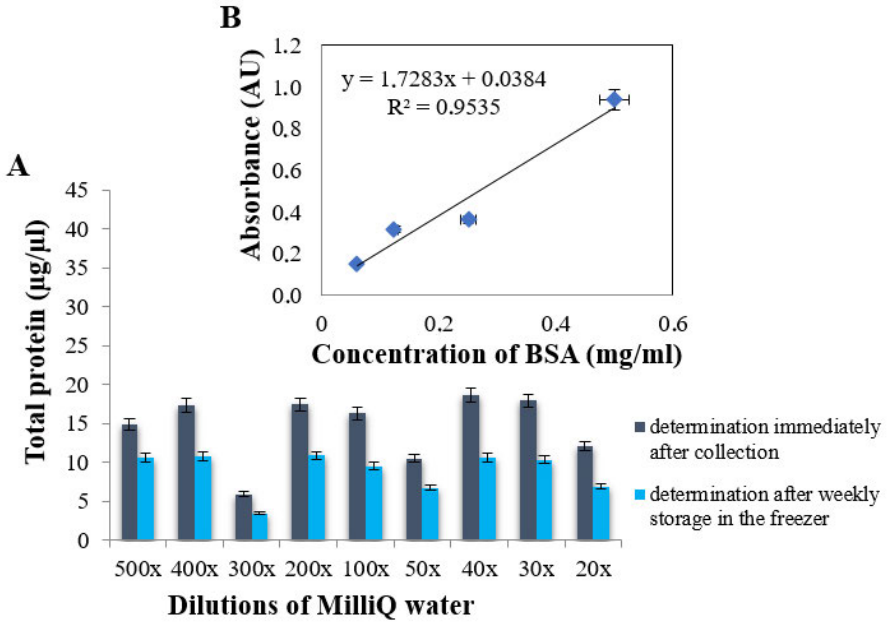
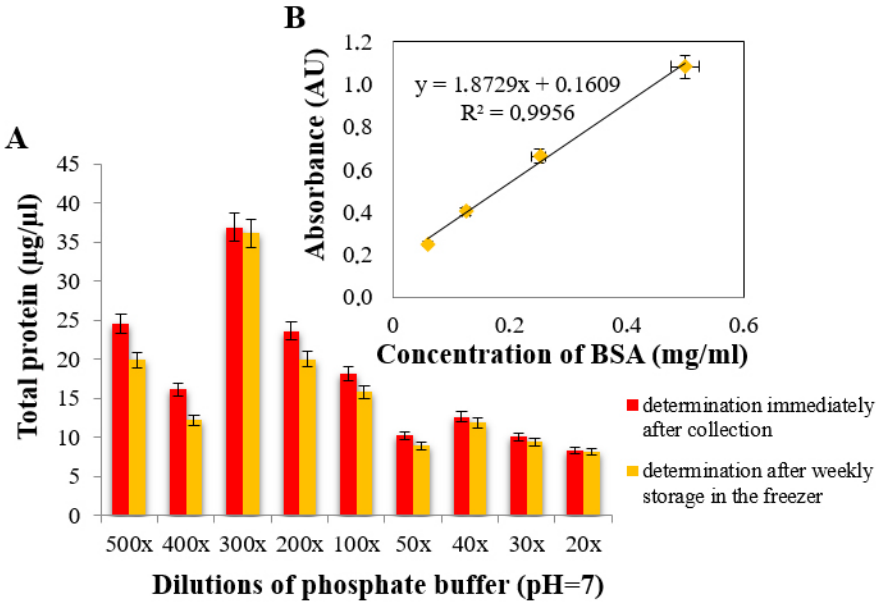


*Figure 2 (**A**) Total protein content in the samples of a bee haemolymph diluted 20–500× in phosphate buffer (pH=7) (determination immediately after the collection and after the one week storage in the freezer). (**B**) The calibration curve of BSA in phosphate buffer (pH=7) in range 0.50 to 0.03 mg/ml.*



An expression of these differences as a percentage is depicted in Figure 3. The haemolymph samples diluted in MilliQ water showed in average a 39% decrease and ones dissolved in the phosphate buffer only 11%. This difference was statistically significant ($p < 0.001$). These comparisons confirm that the samples of haemolymph diluted in the phosphate buffer were more stable than in MilliQ water.

In the case of different sample dilutions, the 200× dilution of both tested diluents was evaluated as optimal. It may seem that a 300x dilution may be optimal, however higher or lower dilutions than 200× were no longer evaluated as optimal due to the extent of the detection limit and the determination of the Infinite M200Pro instrument used. Using 300x dilution would not ensure accuracy and reproducibility of results. Coincidentally, two different bees with extreme protein content were sampled for this dilution, therefore, these markedly different values (both diluents) from the other results are not decisive from the point of view of the dilution optimization.

Figure 3 Comparison of the decrease in the total protein after the one week storage in the freezer for samples dissolved in the MilliQ water and the phosphate buffer (pH = 7); the red oval = optimal dilution.

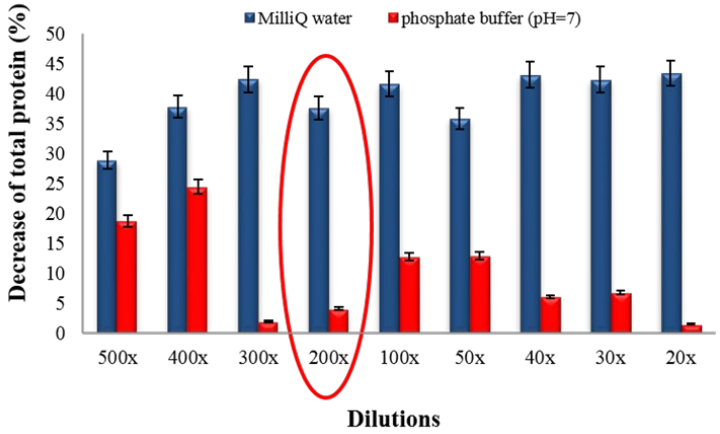
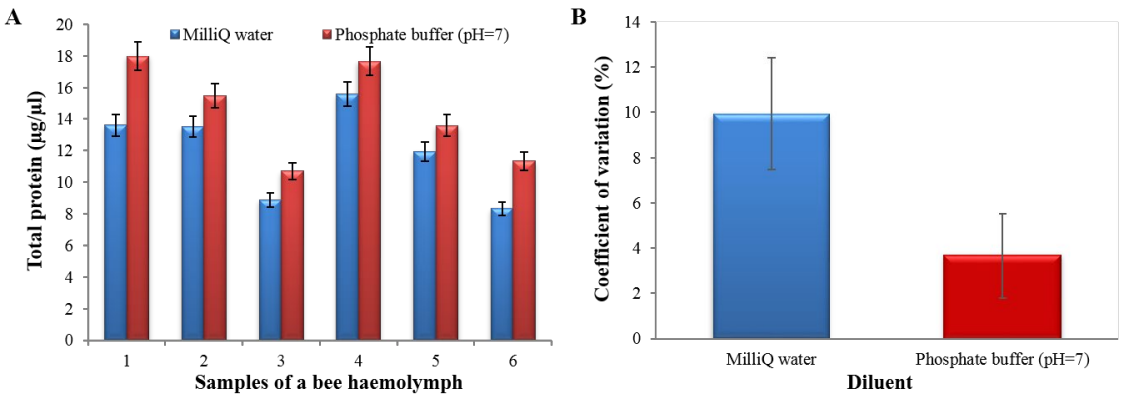


*Figure 4 The paired samples (n = 6) diluted 200× in the MilliQ water and the phosphate buffer (pH = 7); (**A**) the total protein content in the haemolymph; (**B**) average coefficient of variation of the total protein (t-test; p = 0.0006).*



The optimal dilution at 200× was subsequently used in the paired samples (n = 6) for testing of the diluent impact (Figure 4). All haemolymph samples dissolved in the phosphate buffer showed higher yield of protein than from samples diluted in MilliQ water (Figure 4A). The total protein content in the MilliQ water's samples ($x = 11.98$ µg/µl) was significantly (paired t-test; $p < 0.001$) lower by 2.48 µg/µl in average in comparison with the phosphate buffer's samples ($x = 14.45$ µg/µl). The coefficient of variation among three determination (triplets) was significantly ($p = 0.0006$) lower by $v_x = 6.28\%$ in average for phosphate buffer's samples (Figure 4B).

The results confirmed that the phosphate buffer (pH = 7) and the sample dilution 200× were optimal for determination of the total protein in the bee haemolymph due to better protein yield by Bradford's assay. An optimal dilution or diluent have not yet been recommended and are absent also in the protocol by Hartfelder et al. (2013). The protein stability in aqueous solution in our case of experiment means that the all parts of the structure of protein, which consists of the ionisable side chains of aminoacids, which are not currently used in this time for intramolecular moieties, have the same level of chemical charge all the time without any change. Therefore, the reported concentration level by the assay method is the same. The buffer condition provides free ions in higher concentrations, and these can better stabilise the counter ions in the structure of protein by surrounding them. This prevents any change in secondary and ternary structure of bigger proteins, and therefore the assay method reports the same concentration in the same. Contrary, in the water conditions structure of protein may change in the time because the concentration of counter-ions which surrounds the protein structure decrease, and therefore protein founds better conformation which may result from change of structure – folding. After that the reported concentration by the Bradford assay method will be lower because of decrease of count of interaction between the CBB agents with the structure of protein which have to be determined. The works reported the difference between used buffers end the ionic strength which affects the total protein determination when the Bradford´s method is used have been published in some different types of the samples (Aoyama 2006, Sathe et al. 2009, Silverio et al. 2012).

The physiological status of a bee is dependent on the level of juvenile hormone (JH) in the haemolymph (Robinson 1992). Long-living workers (e.g.: winter generation) show the low JH level in the haemolymph which is associated with 2-4times higher level of the total protein in the haemolymph in comparison with summer bees, i.e. short-living bees (Fluri et al. 1982). The quantification of the total protein in the haemolymph is an easy practicable method (Hartfelder et al. 2013) in comparison with the JH quantification (Huang et al. 1995). Therefore, the results can slightly contribute to the refinement of research in the honeybee physiology (e.g.: division of the labour or development of the winter bees).

On the other hand, when any other specific protein could be considered and studied as a marker it is absolutely necessary to recalculate its concentration on total protein. Such approach is common in laboratory analysis for higher animals and plants, but it is much more complicated to transfer it for tiny samples as in case of bees.

CONCLUSION

The results confirm that determination of the total protein in a bee haemolymph is optimized with dilution 200× in the phosphate buffer (pH = 7). This study indicate that sample of haemolymph in the phosphate buffer and stored in -18 °C can be analysed one week later due to insignificant decrease of the total protein content. These findings could thus contribute to the methodical area of assaying the haemolymph parameters in bees.

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