

THE COMPARISON OF CONVENTIONAL AND W.H.O. METHODS FOR PROTEIN DETERMINATION OF CHENOPODIUM ALBUM ALLERGENIC EXTRACT

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ABSTRACT

In present study different protein measurement methods are evaluated on the allergenic extract of *Chenopodium album* pollen, which was previously prepared in our laboratory and applied in skin prick testing in comparison with a commercial extract. The protein content of similar amounts of these two extracts which had caused similar skin reactions were measured with different protein assays such as protein nitrogen unit, Lowry procedure, ultra-violet absorption, and base catalyzed hydrolysis and ninhydrin (B.H.N.). The latter is recommended by World Health Organization (W.H.O.) in order to determine total protein of standard allergenic extracts. Our study indicated some differences between protein amounts measured by the various procedures. According to W.H.O. reports regarding the advantages of B.H.N. assay, due to good correlation with biological activity of allergenic extracts, we also found that this method can indicate the potency of extracts much more precisely.

Keywords: *Chenopodium album*, protein assay, allergenic extracts.

INTRODUCTION

Extracts of some pollens that elicit allergic reactions in sensitive individuals are purified for diagnosis and therapy of human allergic diseases. Although such extracts are complex mixtures of many substances, the well-characterized allergens that are responsible for these immediate hypersensitivity reactions are all proteins.⁽⁶⁾ The measurement of the allergenic activity along with the total protein content of allergenic extracts would provide a measure of the specific activity of allergens. The total protein may therefore be an important parameter for the control of product consistency.⁽⁴⁾

Most of standard methods for total protein determination (e.g. Lowry, dye binding protein, biuret, protein N unit assay, total kjeldahl) when applied to allergenic extracts exhibit problems that include interfering sub-

stances, lack of an appropriate protein standard, or lack of sensitivity.⁽⁹⁾

For several decades the only method to standardize allergenic extracts was the measurement of nitrogen in the extract by phospho-tungstic acid precipitation. In this method results were expressed as protein nitrogen units (PNU), which 1 mg of nitrogen is equal to 100000 PNU.

Although many commercial allergic extracts are still marketed with PNU labeling, the PNU content often correlates poorly with the allergenic potency of an extract. This is particularly true when allergens constitute only a small proportion of the total protein in the extract.

In this study, we compared the conventional methods of detection and quantification of proteins, with the WHO recommendation for *Chenopodium album*

Protein Determination of Allergenic Extract

(ch.a.) allergenic extract.

MATERIALS AND METHODS

1- The preparation of pollen extract: Collection of pollens and preparation of extract were done according to our previous study,⁽⁶⁾ briefly as follow: The pollen grains were collected from the flowering *Chenopodium album* in Karadj region. Collected pollens were immediately vacuum dried at 35°C. Mechanical sieving was later undertaken with 3mm, 0.5mm, 160 µm, 80 µm, and 40 µm, mesh sieves. Microscopic control was then undertaken to ensure that the purity of the pollens was at least 98%. Allergenic extract was then prepared with some modifications. Ten grams of pollen was defatted with ether, dried, and then extracted in 100ml of 0.02M phosphate buffered saline, pH = 7.4. The mixture was stirred overnight at 4°C. The suspension was filtered through filter paper, dialysed against PBS, and sterilized by 0.22 µm filtration.

2- Selection of samples: Two samples, one from homemade extract of Ch. a. and another from D.H.S. company which had similar skin reactions (weal and flare reaction in a particular allergic individual), were examined for protein quantification by different assay methods. The measurement of protein contents of these two samples was repeated five times and the average values were considered as the final results.

3- U.V. detection: According to earlier reports,⁽²⁾ the absorbance of the mixture was read at 280 and 260 nm, and then calculated the approximate protein concentration using following equation:

$$\text{mg/ml of protein} = (1.55 A_{280}) - 0.77 A_{260}$$

4- Protein Nitrogen Unit (PNU): The amount of protein can be determined by the calculation from total kjeldal N or the PNU (100000 PNU=1 mg N) assuming 16% N content in the proteins. In this method the samples of proteins are precipitated with an organic acid such as trichloroacetic acid. The non protein nitrogen is removed with the supernatant. The protein pellet is digested in H₂SO₄ with heat (340°C to 360°C) and a catalyst. The ammonium borate formed is then titrated with a standard solution of HCl to determine the amount of nitrogen in the original protein solution.⁽¹⁾

5- Modified ninhydrin assay (W.H.O. recommendation): Ninhydrin reagent is freshly prepared by mixing equal volumes of stannous chloride dihydrate solution (1.6 mg/ml in 0.7 M citrate buffer, pH = 5.0) and ninhydrin solution (40 mg/ml in 2-methoxy ethanol). Tests samples and standards (bovine serum albumin) containing approximately 1 to 15 µg of protein in 0.1 ml volumes in duplicate forms are placed in 1.5 ml polypropylen centrifuge tubes with snap tops. Proteins are hydrolyzed by the addition of 0.1 ml of 10 M NaOH and incubated overnight at 95°C. Tubes are cooled at room temperature and then sufficient 12 M HCl is added to reduce the pH of the solution to 5.0. After addition of 0.6 ml ninhydrin reagent, the solutions are poured into fresh tubes, which are capped and floated in a boiling water bath for 20 min. Then 0.5 ml from each tube is added to 0.5 ml of diluent in glass tubes. The absorbance in 570 nm of each tube is read and protein content is calculated from BSA standard curve.

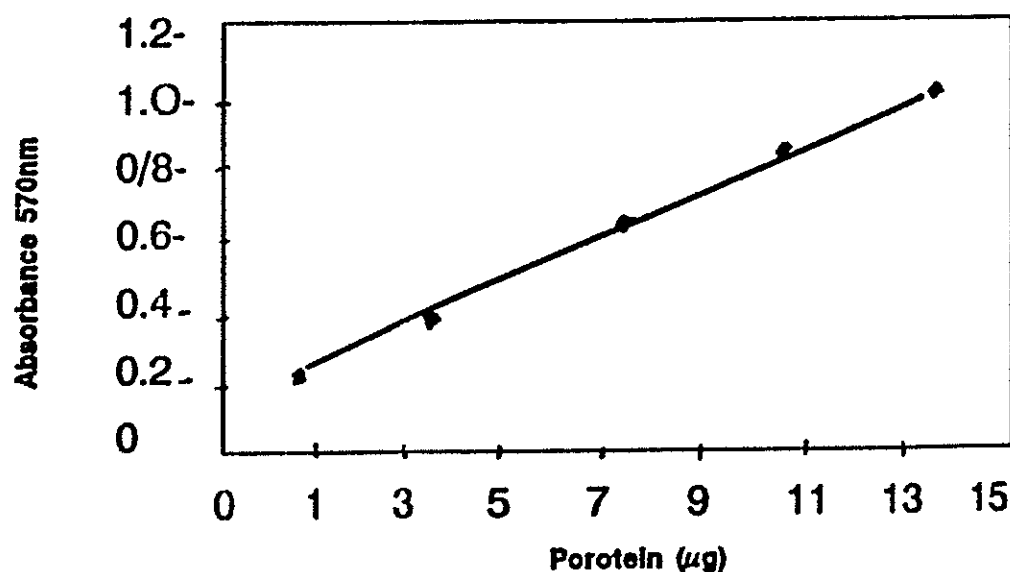


Fig.1. Representative standard curve. The indicated quantities of BSA were assayed by the W.H.O. procedures as described in Material and Methods. Each experimental point is the mean of duplicate determinations.

Table I. The pollen extracts were assayed for protein contents. The mean of five times measurements (mg/ml), and the standard

Protein measurement methods:	WHO Method	PNU	Lowry	U.V. detection
Chenopodium album extract prepared in our lab.	(0.56±0.01)	(0.42±0.015)	(0.73±0.02)	(0.8±0.04)
Ch.a. extract from D.H.S (mg/ml)	(0.55±0.015)	(0.34±0.010)	(0.85±0.017)	(1.01±0.35)

RESULTS

Standard curve of the WHO method: for quantification of protein contents of allergens a five point standard curve with duplicate determinations at each point was performed. A representative standard curve is shown by the solid line in Fig. 1.

Measurement of protein by other methods: Chenopodium album pollen extract prepared in our laboratory and commercial extracts were also assayed for total protein using other methods of protein analysis as described in Method section. The results are shown in comparison with a commercial product (Dome-Hollister-Stier) in Table I.

DISCUSSION

During the past decades, much work on the isolation and characterization of the allergens responsible for human IgE mediated diseases has been accomplished. Allergen quantification is important for many reasons. From a practical standpoint, it is necessary for clinicians to know the allergen contents of the extracts used for diagnosis skin testing and for allergen immunotherapy.⁽⁶⁾ Weed pollens, particularly Chenopodium taxonomically related plants, are common causes of seasonal rhinitis in the world.⁽⁷⁾ In our previous study,⁽⁸⁾ Ch. Album pollens were collected, extracted, semi-purified, and used in skin testing. In the present study, we measured the protein content of Ch. a. extracts, both prepared in our lab and from D.H.S. company according to a modified ninhydrin assay recommended by W.H.O. for standardization of the allergens. The results compared with those of other protein assays. W.H.O. recommendation is based on previous studies, specially the B.H.N. method, introduced by Paul G. in 1988.⁽⁹⁾ According to Paul and his colleagues study, comparison of B.H.N. assay with Lowry, total nitrogen, P.N.U., and amino acid analysis procedures, the advantages of B.H.N. assay in measuring protein content of allergens is brightly proved. Furthermore, this method is being used by F.D.A. licensed manufacturers to provide the protein content to release many standardized extracts.⁽¹⁰⁾ In addition, the precision of the B.H.N. assay reported previously (coefficient of variation = 1%) is comparable to the reported for both the Lowry and dye-binding protein assay.⁽¹¹⁾

The differences that we found in our experiment among various protein assays showed that the results obtained from W.H.O. method has more correlation with skin testing results, than other methods. According to Table I, there is much variation among the results obtained from P.N.U., Lowry, and U.V. detection assays in two examined extracts. As a result, we conclude that expressing the protein content of an allergenic extract by W.H.O. method, because of direct correlation with the biological capacity of the allergen is better for diagnostic purposes.

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