notes on methodology

Determination of protein in adipose tissue extracts

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Summary An accurate and sensitive method for determination of protein in adipose tissue extracts based on a scaled-down version of the method of Lowry et al. (1951, *J. Biol. Chem.* **193,** 265–275) is described. Protein is quantitatively precipitated by trichloroacetic acid with a nonionic detergent as a bulk-increasing substance and the precipitate is extracted with an organic solvent. The interference caused by lipids, sulfhydryl reagents, and several other compounds is eliminated. The accuracy of the determination of dilute reference protein solutions (2–20 μg/ml) is demonstrated.

Supplementary key words nonionic detergent · TCA precipitation · solvent extraction

In the course of the purification of a lipolytic enzyme in rat adipose tissue (1) we observed that the determination of protein in extracts of this organ according to the method of Lowry et al. (2) generally gave erroneously high values. This has previously been observed for tissue extracts containing large amounts of lipids (3). Protein determination in adipose tissue extracts is further complicated by the very small amounts of protein in dilute solution resulting from recent extensive purifications of lipolytic enzymes from this tissue (1, 4, 5) and by the interference of several compounds used in the purifications. These compounds include detergents (6, 7), glycerol (8), sucrose (9), sulfhydryl reagents (10, 11), EDTA (12) and Tris buffer (13). This report describes a simple procedure to remove lipids and other interfering substances in adipose tissue extracts prior to a scaled-down protein determination according to Lowry et al. (2). This is accomplished by a quantitative precipitation of the protein in 10% (w/v) TCA in the presence of detergent followed by extraction of the precipitate with organic solvent. Determination of protein in very dilute solutions, 2–20 μg/ml, is possible.

Material

All chemicals were of reagent grade. Glass-distilled water was always used and all solvents were freshly redistilled. Dithiothreitol (DTT), bovine serum albumin (fraction V) and cytochrome *c* were obtained from Sigma Chemical Co. (St. Louis, Mo.). Human serum from fasting donors was from the local hospital. Triton X-100 was from Packard Instrument Co. (Downers Grove, Ill.) and Nonipol TD 12[1] was from Rexoline Chemicals (Helsingborg, Sweden). EDTA, 2-mercaptoethanol, sucrose, trichloroacetic acid (TCA), sodium hydroxide, Folin-Ciocalteau reagent, buffers, and other reagents were from B. D. H. Laboratory Chemicals (Poole, England). Mixed phospholipids were obtained from a chloroform–methanol lipid extract of rat livers by chromatography on silicic acid.

The reagents were those described by Lowry et al. (2). Reagent A, 2% (w/v) Na_2CO_3. Reagent B, 0.5% (w/v) $CuSO_4 \cdot 5H_2O$ in 1% (w/v) potassium tartrate. For reagent C, 50 ml of Reagent A was mixed with 1 ml of Reagent B. For reagent E, 10 ml Folin-Ciocalteau reagent was diluted with 13 ml of distilled water just before use.

Methods

0.02–5 mg protein/ml. Add to the sample 2–25 μg protein in 5–100 μl detergent (Nonipol TD 12 or Triton X-100) to a final concentration of 0.2% (w/v). Take 0–25 μg aliquots of the reference protein, e.g., 1 mg/ml, in buffer containing 0.2% (w/v) detergent. Mix the precooled (0–4°C) samples with one volume of ice-cold 20% (w/v) TCA in small, narrow glass test tubes (35 × 6/7 mm, round bottom) and keep them on ice. After 60 min sediment the precipitate formed in a small laboratory centrifuge (e.g., Ole Dich type 155 with angle rotor 12 × 0.5 ml, Ole Dich Co., Copenhagen, Denmark) at about 15,000 *g* for 10 min. For large amounts of protein, centrifugation time may be shortened to 2 min. During this centrifugation temperature must not be allowed to exceed 25°C. Decant the supernatant carefully and remove the last drop by holding the test tube upside-down against a filter paper. Wash the precipitate with 500 μl of ice-cold 10% (w/v) TCA and centrifuge. Extract the precipitate with 500 μl of diethyl ether–ethanol 3:1 (v/v) and centrifuge for 5 min (2 min). Repeat the solvent extraction step. Let the protein precipitate dry at 21°C

Abbreviations: EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid.

[1] Nonipol TD 12, a polydisperse preparation of tridecyl polyethoxyethanols with an average molecular weight of 728, similar to the nonionic detergents of the Brij and Lubrol series.

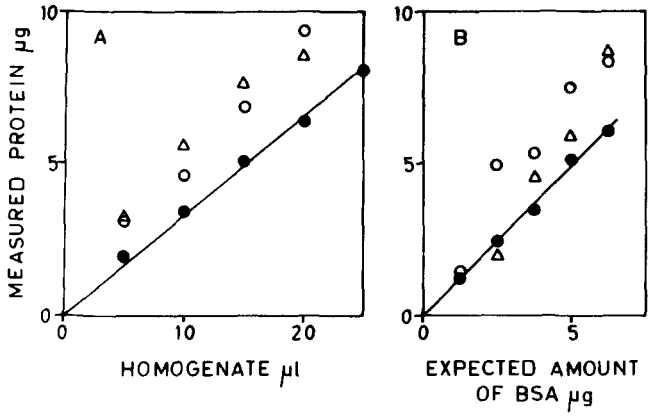

Fig. 1. Protein determination in adipose tissue homogenate. (A) Protein content in 0–25 µl of a 10% homogenate in 0.15 M NaCl and (B) the increase in protein content caused by the addition of 0.25 mg/ml bovine serum albumin (BSA) to the same homogenate. Protein was measured essentially as described by Lowry et al. (2) directly on the homogenate (○), after precipitation in 10% TCA (△) and after TCA precipitation and solvent extraction according to the present method (●).

in a desiccator under reduced pressure. Dissolve the sample in 25 µl of 1 M NaOH at 60°C for 30 min. Ensure that the walls of the test tube are reached by the solution by vigorously shaking the tube. Finally, add 275 µl of Reagent C and 10 min later add 25 µl of Reagent E while shaking the test tube vigorously. Measure the absorbance at 750 nm 30 min later in a 250 µl cuvette with 1.0 cm light path against distilled water. Calculate the protein content by comparison with the reference protein.

2–20 µg protein/ml. Precipitate in triplicate 200 µl of each sample solution to be analyzed. Follow the procedure described above, but wash the precipitates twice with 500 µl 10% (w/v) TCA. Calculate the mean absorbance per 200 µl. Treat and analyze seven standard solutions (e.g., 0, 2, 6, 10, 14, 16, and 20 µg/ml) of the reference protein, freshly made each day, in exactly the same way. Plot absorbance/200 µl solution against µg/ml of the standard protein (see Fig. 2).

Statistics. Simple linear regression analysis was performed according to the method of least squares. Confidence intervals were calculated as described by Snedecor and Cochran (14) for a dependent x value and an independent y value, the protein concentration and absorbance value, respectively.

Results and discussion

Standard curves. A 0–25 µg volume of bovine serum albumin, cytochrome *c*, or human serum in 25 µl of 20 mM phosphate buffer, pH 7.0, determined as described in Methods gave curves almost identical with those obtained by direct determination according to Lowry et al. (2). Recovery of protein during the precipitation, washing and solvent extraction procedures was generally more than 95%. The presence of detergent was found to be essential for a quantitative precipitation of the protein. Its function is probably to increase the bulk of the precipitated material (cf. ref. 4). The concentration of the detergent was not critical; 0.1 or 0.4% (w/v) did not seem to affect the results. The detergent is quantitatively removed during the solvent extraction step. A low temperature (0–4°C) was also essential for the precipitation. The solubilization of the final protein precipitate in 1 M NaOH should be made carefully (cf. ref. 2). The average coefficient of variation for multiple determinations was 3%.

Crude adipose tissue extracts. Protein content was estimated in a 10% (w/v) homogenate of rat adipose tissue in 0.15 M NaCl (**Fig. 1**). When protein was determined directly or after TCA precipitation of the homogenate according to Lowry et al. (2) values were 30–50% higher than those obtained after TCA precipitation and lipid extraction (Fig. 1A). This difference was due to the lipid material removed by the solvent extraction. Test experiments revealed that less than 5% of the total amount of protein was found in the discarded TCA solution and solvent extracts. To further test the accuracy of the method, the increase in protein content after the addition of 0.25 mg/ml bovine serum albumin to the same homogenate was measured. This increase measured by the present method correlated well with the amount expected

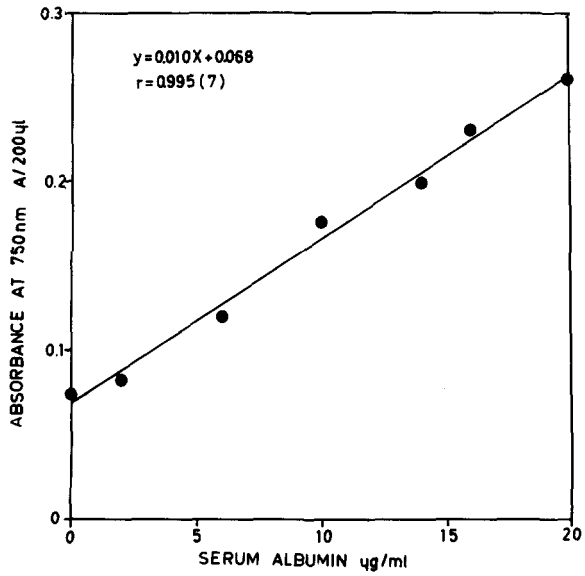

Fig. 2. Calibration curve for dilute solutions of bovine serum albumin, 2–20 µg/ml, in 20 mM phosphate buffer, pH 7.0, containing 0.2% (w/v) Nonipol TD 12, 1 mM EDTA, and 1 mM DTT. Blank values are not subtracted. Each point represents the mean of triplicates.

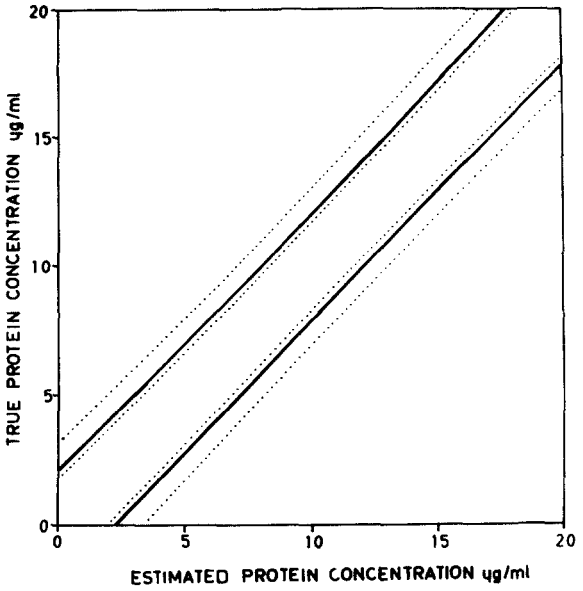

Fig. 3. Accuracy of the determination of dilute protein samples with bovine serum albumin. The figure summarizes the results from eleven calibration curves on different days. The solid curved lines show the median 90% confidence interval for a given estimated concentration together with the range of this parameter (1st and 3rd quartile, dotted lines).

(Fig. 1B). Corresponding values measured directly in the homogenate or after TCA precipitation gave mean values 40% higher than expected. Thus, to obtain an accurate value for protein concentration in crude adipose tissue homogenates, removal of the lipid material as described here was necessary.

Interfering substances. The effect on protein determination of 0.2% (w/v) Triton X-100 or Nonipol TD 12, 10 mM EDTA, 10 mM DTT, 0.1 M 2-mercaptoethanol, 50% (w/v) sucrose, 20% (w/v) glycerol, 0.2 M Tris, or 1 mg/ml mixed phospholipids was eliminated by the pretreatment described. This is of practical importance especially in the case of DTT or 2-mercaptoethanol, where protein determination otherwise would be difficult (10). These findings are in accordance with and extend those of Bensadoun and Weinstein (15).

Dilute protein solutions, 2–20 µg/ml. **Fig. 2** shows a representative calibration curve with 0–20 µg/ml bovine serum albumin in 20 mM phosphate buffer, pH 7.0, 0.2% (w/v) Nonipol TD 12, 1 mM EDTA, and 1 mM DTT. The recovery of protein was >90% as compared to 0–4 µg samples measured directly according to Lowry et al. (2). The interference caused by EDTA and DTT was eliminated. Test experiments with a small protein (cytochrome *c*) and a mixture of proteins (human serum) gave the same results. No difference was seen if Triton X-100 was used instead of Nonipol TD 12 as the bulk-increasing substance during the precipitation step.

In order to evaluate the accuracy in the determination of a 2–20 µg/ml protein sample, eleven calibration curves (cf. Fig. 2) with bovine serum albumin were made. For each curve with seven values, each value being based on the mean of three determinations, the 90% confidence interval was calculated. **Fig. 3** illustrates the results of these calculations. The use of several separate standard solutions with varying protein concentrations was employed in order to ensure that a quantitative protein precipitation, which is the critical step, was achieved over the entire protein concentration range. Triplicates of 200 µl of a dilute sample solution was chosen as an optimal compromise between reliability and consumption of sample. If n determinations are made on the same sample solution with separate calibration curves, the calculated variation coefficient would decrease by an approximate factor of $1/\sqrt{n}$. When larger volumes (e.g., 1–2 ml) of the protein sample are to be precipitated, this could be done in larger test tubes by adding one-fourth volume of 50% (w/v) TCA or by successive precipitation of 200 µl aliquots in a single small test tube.

In our hands this modification of the method of Lowry et al. (2) has proven a useful tool for serial determinations of protein in adipose tissue extracts and during various stages of enzyme purification from such tissue. A similar method using protein precipitation in the presence of an ionic detergent but employing no solvent extraction has recently been described (15). When dealing with adipose tissue extracts, however, it is essential to remove lipids from the protein precipitates by solvent extraction in order to obtain a correct measure of protein concentration.

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