PROTOCOL

Ultrafast Protein Determinations Using Microwave Enhancement

Robert E. Akins[*,1] and Rocky S. Tuan[2]*

Abstract

We present here microwave-based modifications of standard protein assays that dramatically reduce the time required to determine protein concentrations. Typical protein determinations involve incubation times ranging from 15–60 min. Microwave irradiation of specimens reduces this time requirement to 10–20 s without compromising accuracy or reliability. The remarkable speed with which protein determinations may be carried out using microwave enhancement greatly simplifies general laboratory procedures that depend on the estimation of protein concentrations.

Index Entries: Biuret reaction; Lowry assay; Folin-Ciocalteau phenol reagent; bicinchoninic acid; BCA; microwave; protein assay.

1. Introduction

In this work, we describe modifications of existing protein assays that take advantage of microwave irradiation to reduce assay incubation times from the standard 15–60 min down to just seconds *(1)*. Adaptations based on two standard protein assays will be described:

1. The classic method of Lowry et al. *(2)*, which involves intensification of the biuret reaction through the addition of Folin-Ciocalteau phenol reagent; and
2. The recently developed method of Smith et al. *(3)*, which involves intensification of the biuret assay through the addition of bicinchoninic acid (BCA).

Performing incubations in a 2.45–GHz microwave field (i.e., in a microwave oven) for 10–20 s results in rapid, reliable, and reproducible protein determinations. We have provided here background information concerning protein assays in general and the microwave enhanced techniques in particular. The use of microwave exposure as an aid in the preparation of chemical and biological samples is well established; interested readers are directed to Kok and Boon *(4)* for excellent discussions concerning the application of microwave technologies in biological research.

1.1. Protein Estimations

There are several spectrophotometric techniques commonly used for the estimation of proteins. Generally, methods of protein estimation involve detection based on:

1. Absorption of light by the amino acid side chains or peptide bonds of the protein;
2. Interaction of a specific compound with chemical moieties in the protein forming an optically detectable compound; or
3. Production of a detectable reaction product owing to the presence of protein.

The method of Warburg and Christian *(5)* takes advantage of the absorption of UV light by the amino acid constituents of proteins, especially tyrosine and tryptophan. The concentration of protein may be determined in the presence of contaminating nucleic acids by comparing absorbance values at 260 and 280 nm determined for unknown samples with those determined for

*Author to whom all correspondence and reprint requests should be addressed. [1]Department of Medical Cell Biology, Nemours Research Programs, A.I. duPont Institute, PO Box 269, Wilmington, DE 19899; [2]Department of Orthopedic Surgery, Thomas Jefferson University, Philadelphia, PA.

known standards of purified yeast enolase and nucleic acid. As an example, a pure preparation of protein will have a A_{280}/A_{260} ratio of 1.75, and, for a 1-cm cuvet, the protein concentration (in mg/mL) will be equivalent to the $A_{280} \times 1.118$ (*see* ref. *6*). This method is simple and extremely rapid; however, it is based on the assumption that all proteins possess the same extinction coefficient as the standard protein. The results are, therefore, potentially misleading, and the technique may be best suited to estimations of relative quantities in similar preparations.

The method of Bradford *(7)* is based on the binding of a specific dye (Coomassie brilliant blue) to proteins in solution. Dye binding results in a colorimetric change, and the amount of protein can be estimated spectrophotometrically based on the absorbance of the resulting solution. This assay is rapid; however, its sensitivity is somewhat limited. Additionally, the dye:protein interaction is not specific and is influenced by the net charge of specific amino acid residues. Also, the presence of other compounds, including some detergents commonly used in protein preparations, may result in substantially inaccurate protein estimations.

Other methods are based on the well-known biuret reaction *(8)*. In the biuret reaction, $CuSO_4$ is prepared in a Rochelle salt solution at alkaline pH. When combined with proteins or amino acids, this reagent changes color, and concentrations may be estimated based on the absorbance at 550 nm of the resulting solution. Several modifications of the biuret reaction are in general use, including the method of Lowry et al. (*2;* Note 1) and the method of Smith et al. (*3;* Note 2). These adaptations increase the detectability of the reduced copper ions through the addition of Folin-Ciocalteau phenol reagent or BCA, respectively.

The biuret-based assays are sensitive, accurate, and relatively simple to perform; however, they require on the order of 1/2–1 h to complete. In some instances, this time requirement and the delays it can cause may compromise the overall efficiency of experimental or diagnostic procedures. We have shown that exposure of samples in biuret-based assays to microwave fields generated in household microwave ovens results in a dramatic reduction in the time required to perform routine protein assays *(1)*.

1.2. Microwave Assay

Household microwave ovens expose materials to nonionizing electromagnetic radiation at a frequency of about 2.45 GHz (i.e., 2.45 billion oscillations/s). Such exposures have been applied to a myriad of scientific purposes including tissue fixation, histological staining, immunostaining, PCR, and many others. The practical and theoretical aspects of many microwave techniques are summarized by Kok and Boon *(4)*, and the reader is directed to this reference for excellent discussions concerning general procedures and theoretical background.

Microwave ovens are conceptually simple and remarkably safe devices (*see* Note 3). Typically, a magnetron generator produces microwaves that are directed toward the sample chamber by a wave guide. The beam is generally homogenized by a "mode-stirrer," consisting of a reflecting fan with angled blades that scatter the beam as it passes. The side walls of the chamber are made of a microwave reflective material, and the microwaves are thereby contained within the defined volume of the oven (*see* Note 4). Specimens irradiated in a microwave oven absorb a portion of the microwave energy depending on specific interactions between the constituent molecules of the sample and the oscillating field.

As microwaves pass through specimens, the molecules in that specimen are exposed to a continuously changing electromagnetic field. This field is often represented as a sine wave with amplitude related to the intensity of the field at a particular point over time and wavelength related to the period of oscillation. Ionically polarized molecules (or dipoles) will align with the imposed electromagnetic field and will tend to rotate as the sequential peaks and troughs of the oscillating "wave" pass. Higher frequencies would, therefore, tend to cause faster molecular rotations. At a point, a given molecule will no longer be able to reorient quickly enough to align with the rapidly changing field, and it will cease spinning. There

exists, then, a distinct relationship between microwave frequency and the "molecular-size" of the dipoles that it will affect. This relationship is important, and at the 2.45–GHz frequency used in conventional microwave ovens, only small molecules may be expected to rotate; specifically, water molecules rotate easily in microwave ovens but proteins do not (Note 5).

For most microwave oven functions, a portion of the rotational energy of the water molecules in a sample dissipates as heat. Since these molecular rotations occur throughout exposed samples, microwave ovens provide extremely efficient heating, and the effects of microwaves are generally attributed to changes in local temperature. At some as yet undetermined level, however, microwave exposure causes an acceleration in the rate of reaction product formation in the protein assays discussed here. This dramatic acceleration is independent of the change in temperature, and our observations have suggested that microwave-based heating is not the principal means of reaction acceleration.

1.3. Microwave Enhanced Protein Determinations

Modifications of the procedures of Lowry *(2)* and Smith *(3)* to include microwave irradiation result in the generation of linear standard curves. Figure 1 illustrates typical standard curves for examples of each assay using bovine serum albumin (BSA). Both standard curves are linear across a practical range of protein concentrations.

One interesting difference between the two microwave enhanced assays concerns the relationship between irradiation time and assay sensitivity. In the *DC Protein Assay*, a modification of Lowry's *(2)* procedure supplied by Bio-Rad Laboratories, Inc. (Hercules, CA), illustrated in Fig. 1, a colorimetric end point was reached after 10 s of microwave irradiation; no further color development occurred in the samples. This end point was identical to that achieved in a 15-min room temperature control assay.

The *BCA* Protein Assay*, a version of Smith's assay *(3)* supplied by Pierce Chemical Co. (Rockford, IL), afforded some flexibility in assay sensi-

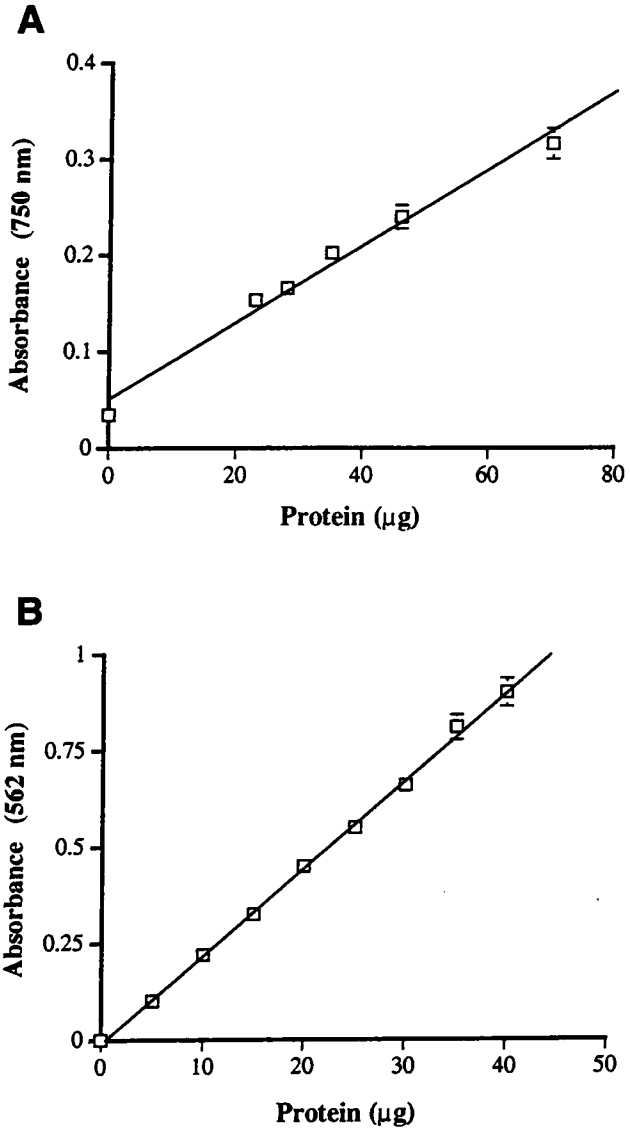

Fig. 1. Typical standard curves for microwave Lowry and BCA assays. Standard curves were generated with microwave protocols using BSA (Sigma) dissolved in water. BSA samples in 100-µL vol were prepared in triplicate for each assay and were combined with reagent as described in Notes 1 and 2. Tubes and a water load (total vol 100 mL, *see* Note 7) were placed in the center of a microwave oven. Samples for the Lowry assay were irradiated for 10 s; samples for the BCA assay were irradiated for 20 s. Results in **A** show a linear standard curve generated using a microwave Lowry assay. Results in **B** show a linear standard curve generated using a microwave BCA assay. Values presented are means ± SD.

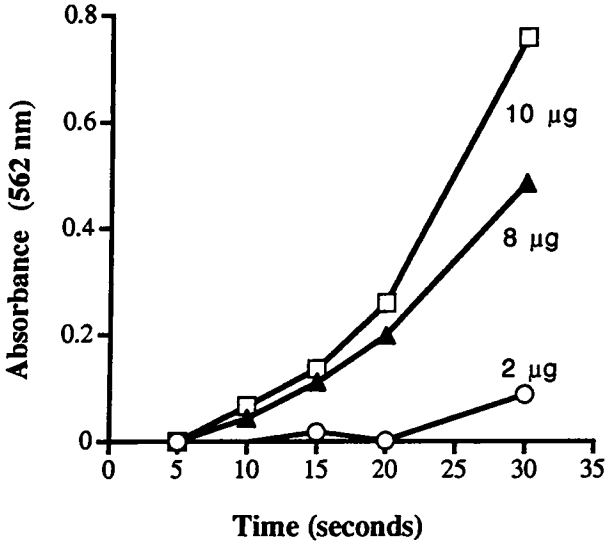

Fig. 2. Effect of Increasing microwave irradiation time on the BCA assay. Three amounts of BSA (Sigma) were prepared in water: 10 µg/tube (□), 8 µg/tube (▲), 2 µg/tube (○). Each time point was determined from triplicate samples in a single irradiation trial with the water load replaced between determinations. Each assay time resulted in the generation of a linear standard curve; the slope of each standard curve increased as a function of irradiation time. Values were normalized to the 5-s time point and presented as means ± SD.

tivity because the formation of detectable reaction product was a function of the duration of microwave exposure. Figure 2 shows the rate of reaction product formation for three different concentrations of BSA as a function of microwave exposure in a BCA assay. Absorbance values increased for each BSA concentration as a second order function of irradiation time. A linear standard curve could be generated from BSA dilutions that were irradiated for any specific time; longer irradiation times yielded more steeply sloped standard curves. In practice, then, the duration of microwave exposure can be selected to correspond to a desired sensitivity range with longer times being more suitable for lower protein concentrations. In contrast to the *DC Protein Assay*, the BCA microwave procedure described here is more sensitive than a standard, room temperature assay, and we have used it for most applications (*see* Note 6).

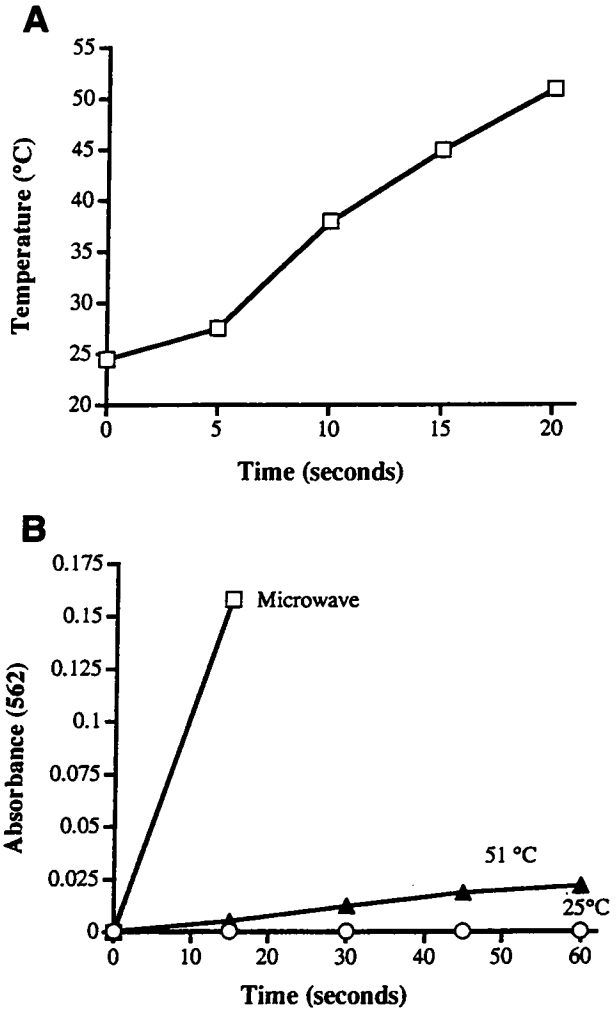

Fig. 3. Comparison of microwave irradiation with incubation at elevated temperature. **(A)** The change in temperature of BCA assay samples containing bovine serum albumin (BSA, Sigma). Temperatures reached 51°C during a typical 20-s irradiation. Since the assays are carried out in an open system, temperatures near 100°C would cause sample boil-over and should be avoided. **(B)** Development of reaction product under three different conditions: microwave irradiation (□), incubation at 51°C (▲), and incubation at 25°C (○). Incubation at 51°C, the maximal temperature reached during a 20-s irradiation, did not mirror microwave irradiation.

Figure 3 illustrates that the dramatic effects of microwave exposure cannot be mimicked by external heating. These results are surprising since microwave effects are generally attributed to

increases in temperature. It is not clear at what level(s) microwaves interact with the biochemical processes involved in protein estimation; however, the acceleration is possibly related to an alteration in solvent/solute interactions. As the solvent H_2O molecules rotate, specific structural changes may occur in the system such that interactions between solvent and solute molecules (or among the solvent molecules themselves) tend to enhance the chemical interactions between the protein and the assay components to accelerate the rate of product formation. For example, water molecules rotating in a microwave field may no longer be available to form hydrogen bonds within the solvent/solute structure. Clearly, the nature and mechanism of nonthermal microwave effects need to be studied further.

2. Materials

2.1. Lowry Assay

Lowry reagents are available from commercial sources (*see* Note 1). Assay reagents were routinely purchased from Bio-Rad Laboratories in the form of a detergent-compatible Lowry kit (*DC Protein Assay*). Assay reactions were typically carried out in polystyrene Rohren tubes (Sarstedt, Inc., Newtown, NC). Tubes were placed in a plastic test tube rack at the center of a suitable microwave oven *(see the following)* along with a beaker containing approx 100 mL of H_2O (*see* Note 7).

2.2. BCA Assay

BCA protein reagent is available from commercial sources (*see* Note 2). Assay reagents were routinely purchased from Pierce Chemical Co. in the form of a *BCA* Protein Assay* kit. As with the Lowry assay, reactions were typically carried out in polystyrene Rohren tubes (Sarstedt). Tubes were placed in a plastic test tube rack at the center of a suitable microwave oven *(see the following)* along with a beaker containing approx 100 mL of H_2O (*see* Note 7).

3. Method

3.1. Sample Preparation

Sample preparation should be carried out as specified by the manufacturers. Generally, samples are solubilized in a noninterfering buffer (*see* Note 5) so that the final protein concentration falls within the desired range (*see* Note 6). Samples should be either filtered or centrifuged to remove any debris prior to protein determination. Place 100 µL of each sample and of a series of standard protein dilutions into separate polystyrene Rohren tubes. Place these tubes at even intervals into a plastic test tube rack. For a Lowry assay (described in Note 1), add 500 µL of the assay stock solution (a combination of Reagents I and II) to each tube; mix well and add 4 mL of Reagent III. For a BCA assay (described in Note 2), prepare the assay stock solution by combining 50 mL of Reagent I with 1 mL of Reagent II; add 2.0 mL of assay stock solution to each standard and sample tube. The test tube rack containing all samples and standards should be placed into the center of the microwave chamber.

3.2. Selection of Microwave Oven

Microwave ovens differ substantially in their suitability for these assays. Desirable attributes include fine control of irradiation time, a chamber size large enough to easily accommodate the desired number of samples, and a configuration that results in a homogenous field of irradiation so that all samples within the central volume of the oven receive a uniform microwave dose (Note 4). Samples should be placed in a nonmetallic test tube rack in the center of the oven. A volume of room temperature water is included in the oven chamber as well so that the total amount of fluid (samples + additional water) is constant from one assay to another (Note 7).

3.3. Sample Irradiation

Once the samples are placed into the center of the microwave chamber, close the door and irradiate the samples. Using the *DC Protein Assay* , a 10-s irradiation was optimal as a replacement for the standard 15–min incubation. Using the *BCA* Protein Assay*, a 20-s irradiation has proved adequate in most situations. We have found it most convenient to use the highest setting on the microwave oven and to control exposure using an accurate timer.

3.4. Reading and Interpreting Assay Results

After irradiation, the absorbance of each sample and standard should be determined spectrophotometrically. Samples from Lowry assays should be read at 750 nm and samples from BCA assays should be read at 562 nm. A standard curve of absorbance values as a function of standard protein concentration can be generated easily and used to determine the protein levels in the unknown samples. It is recommended that standard curves be generated along with each assay to avoid any difficulties that may arise from differences in reagents or alterations in total microwave exposure.

4. Summary

In summary, the microwave BCA protein assay protocol is as follows:

1. Combine samples and BSA standards with BCA assay reagent in polystyrene tubes;
2. Place samples into an all plastic test tube rack in the center of a microwave oven along with a beaker containing a volume of room temperature water sufficient to make the total volume of liquid in the chamber 100 mL;
3. Irradiate samples for 20 s on the highest microwave setting; and
4. Measure A_{562} for each sample and determine protein concentrations based on a BSA calibration curve.

The microwave Lowry assay protocol is virtually identical:

1. Combine samples and Lowry assay reagents in polystyrene tubes;
2. Place samples into an all plastic test tube rack in the center of a microwave oven along with a beaker containing a volume of room temperature water sufficient to make the total volume of liquid in the chamber 100 mL;
3. Irradiate samples for 10 s on the highest microwave setting; and
4. Measure A_{750} for each sample and determine protein concentrations based on a BSA calibration curve.

Microwave protein assays are suitable for all situations where standard assays are presently used. The ability to determine accurate protein concentrations in so little time should greatly facilitate routine assays and improve efficiency when protocols require protein determination at multiple intermediate steps. Similar microwave techniques have also been applied as time-saving and efficiency-enhancing procedures by several authors (*see* ref. *4*). We have used microwave assays to generate chromatograms during protein purification and for general protein determinations (e.g., before electrophoretic analysis). The assays consistently yield reliable results that are comparable to those obtained by standard protocols. The modifications we present here are very easily adapted to most commercially available microwave ovens and, in the case of the BCA assay, can be adjusted to cover a wide range of protein concentrations. Since the duration of the assays is so short, it is possible to try several irradiation times and water loads to select the specific conditions required by the particular microwave and samples to be used. Microwave enhanced protein estimations should prove to be extremely useful in laboratories currently doing standard Smith *(3)* or Lowry *(2)* based protein determinations.

5. Notes

1. The method of Lowry et al. *(2)* involves three basic reagents. Reagent I is a 2% solution of Na_2CO_3 in 0.1*N* NaOH, Reagent II is a 0.5% solution of $CuSO_4 \cdot 5H_2O$ in 1% sodium/potassium tartrate (Rochelle salt, $NaKC_4H_4O_6 \cdot 4H_2O$), and Reagent III is Folin-Ciocalteau phenol reagent. The preparation of Folin-Ciocalteau reagent is described in Dawson et al. *(9)*: combine 100 g of $Na_2WO_4 \cdot 2H_2O$ with 25 g of $NaMoO_4 \cdot 2H_2O$ in 700 mL H_2O, add 100 mL concentrated HCl and 50 mL 85% H_3PO_4, and reflux for 10 h; add 150 g Li_2SO_4, 50 mL H_2O, and about 250 mL Br_2; boil for approx 15 min (to remove excess Br_2); after cooling, add H_2O to 1L and filter through Whatman (Maidstone, UK) #1 paper; store in the refrigerator; prior to use, dilute with water to make a 1*M* acid solution. To perform the assay, combine 50 mL of Reagent I with 1 mL of Reagent II to prepare an assay stock. To 5ml of assay stock, add 1 mL of protein solution containing 25–500 mg of protein. Mix well. Add 0.5 mL of Reagent III

and vortex immediately. After 30 min, read the absorbance at 750 nm, and compare the measured value to a suitable (not necessarily linear) standard curve generated from known protein solutions.

The authors suggest that commercial reagents be used for routine protein estimations by the Lowry method. Kits are available from a variety of sources including Sigma Chemical Co. (St. Louis, MO), Bio-Rad Laboratories, and Pierce Chemical Co. These kits have the advantages of better reagent stability, improved assay compatibility, and a reduced number of steps involved. A few caveats should be noted. For example, solutions of different proteins at the same absolute concentration may result in different absorbance values owing, in part, to their amino acid compositions. Also, several commonly used biochemical reagents interfere with the assay. Users are directed to the manufacturers for specific information concerning the various reagent formulations. It should be pointed out that extended microwave irradiation of samples in the *DC Protein Assay* should be avoided as it may result in precipitation of some assay components.

2. The method of Smith et al. *(3)* involves two aqueous reagents. Reagent I is a solution of 1% bicinchoninic acid (BCA), 2% $Na_2CO_3 \cdot H_2O$, 0.16% $Na_2C_4H_4O_6 \cdot 2H_2O$, 0.4% NaOH, and 0.95% $NaHCO_3$ adjusted to pH 11.25. Reagent II is 4% $CuSO_4 \cdot 5H_2O$. To perform the assay, 50 mL of Reagent I is combined with 1 mL of Reagent II to form an assay stock. The assay stock is combined with a protein solution in a 20–1 ratio (e.g., 2 mL assay stock for 100 mL of protein for a standard spectrophotometer cuvet, or 200 mL assay stock for 10 mL of protein for a 96-well microtiter plate). Three standard assay incubations are recommended by Pierce: 2 h at room temperature, 30 min at 37°C, and 30 min at 60°C. The assay incubation selected depends, in part, on the desired sensitivity.

   Although the preparations involved are much simplified for the Smith/BCA assay compared to the Lowry/Folin-Ciocalteau-phenol assay, the authors still suggest that commercial reagents be used for routine protein estimations. A variety of kits are available from Pierce that allow considerable latitude in assay design. A few caveats should be noted. For example, solutions of different proteins at the same absolute concentration may result in different absorbance values owing, in part, to their amino acid compositions. Also, several commonly used biochemical reagents interfere with the assay. Users are directed to the manufacturers for specific information concerning the various reagent formulations.
3. Although microwave radiation is nonionizing, precautions should be taken to avoid direct irradiation of parts of the body. Microwaves can penetrate the skin and cause significant tissue damage in relatively short periods of time. Most contemporary microwave ovens are remarkably safe and leakage is unlikely; however, periodic assessment of microwave containment within the microwave chamber should be carried out. Perhaps more dangerous than radiation effects are potential problems caused by the rapid heating of irradiated samples. Care should be taken when removing samples from the microwave chamber to avoid getting burned, sealed containers should not be irradiated as they may explode, and metal objects should be excluded from the chamber to avoid sparking. Users should consult their equipment manuals and institute safety offices prior to using microwave ovens. A detailed discussion of microwave hazards is included in ref. *4*.
4. Microwave ovens use a nominal frequency of 2.45 GHz. The energy put into the microwave chamber is actually a range of frequencies around 2.45 GHz. As the waves in the oven reflect off of the metal chamber walls, "hot" and "cold" spots may be set up by the constructive and destructive interference of the waves. The positions of these "hot" and "cold" spots is a function of the physical design of the oven chamber and the electrical properties of the materials contained in the oven. The unevenness of microwave fields can be minimized by mode stirring *(see the preceeding)*, appropriate oven configuration, or by rotating the specimen in the chamber during irradiation. If the design of a particular microwave oven does not provide a relatively uniform irradiation volume, it may not be useful for the assays outlined here.

The suitability of a particular microwave oven may be tested easily using a number of tubes with known concentrations of protein. Uneven irradiation patterns will be detected by significant differences in the color development for a given protein concentration as a function of position within the oven chamber. We have had success with several microwave ovens including a 0.8 cu ft, 600W, General Electric oven, model JEM18F001 and a 1.3 cu ft, 650W, Whirlpool oven, model RJM7450.

5. The frequency used in household microwave ovens is actually below the resonance frequency for water. Above the 2.45 GHz used in household microwave ovens, water molecules are capable of rotating faster and of absorbing substantially more microwave energy. Too much absorption, however, is undesirable. It is possible that the outer layers of a sample may absorb energy so efficiently that the interior portions receive substantially less energy. The resulting uneven exposure may have adverse effects.

   It is important to note that molecules other than water may absorb microwave energy. If a compound added to a microwave enhanced assay absorbs strongly near 2.45 GHz, uneven sample exposures may result because of the presence of the compound. In addition, compounds that are degraded or converted during a microwave procedure, or compounds that have altered interactions with other assay components during irradiation, may substantially affect assay results. Although it may be possible to predict which materials would interfere with a given assay by considering the relevant chemical and electrical characteristics of the constituent compounds, potential interference is most easily assessed empirically by directly determining the effects of a given additive on the accuracy and sensitivity of the standard microwave assay.
6. Assay sensitivity may be improved by increasing irradiation time. The time required may be determined quickly by using BSA test solutions in the range of protein concentrations expected until desirable A_{562} values were obtained. By increasing microwave exposure time, it is possible to substantially increase assay sensitivity while keeping irradiation times below 60 s. The ease with which sensitivity may be adjusted within extremely short time frames places the microwave BCA assay among the quickest, most flexible assays available for protein determinations.
7. The addition of a volume of water to the microwave chamber, such that the total volume contained in the microwave chamber is constant from one assay to the next, allows irradiation conditions to be controlled easily from assay to assay. The additional water acts as a load on the oven and absorbs some of the microwave energy. Since the total amount of water remains constant from one assay to the next, the amount of energy absorbed also remains constant. The time of irradiation, therefore, becomes independent of the number of samples included in the assay.

Acknowledgments

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References

1. Akins, R. E. and Tuan, R. S. (1992) Measurement of protein in 20 seconds using a microwave BCA assay. *Biotechniques* **12,** 496–499.
2. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.***193,** 65–275.
3. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. *Analyt. Biochem.* **150,** 76–85.
4. Kok, L. P. and Boon, M. E. (1992) *Microwave Cookbook for Microscopists.* Coulomb, Leyden, Leiden, Netherlands.
5. Warburg, O. and Christian, W. (1941) Isolierung und kristallisation des garunsferments enolase. *Biochemische Zeitschrift* **310,** 384–421.
6. Layne, E. (1957) Spectrophotometric and turbidimetric methods for measuring proteins. *Meth. Enzymol.* **3,** 447–454.
7. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* **72,** 248–254.
8. Gornall, A. G., Bardawill, C. J., and David, M. M. (1949) Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177,** 751.
9. Dawson, R. M. C., Elliott, D. C., Elliott, W. H., and Jones, K. M. (1969) *Data for Biochemical Research.* 2nd ed. Oxford University Press, London, p. 618.