

Different Effects of Microwave Energy and Conventional Heat on the Activity of a Thermophilic β -Galactosidase From *Bacillus acidocaldarius*

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The effect of microwave ($f = 10.4$ GHz) irradiation on a thermostable enzyme was experimentally tested, showing that irreversible inactivation is obtained. Enzymatic solutions (500 μ l, with concentrations between 10–100 μ g/ml) were exposed at 70 °C, at SAR levels of 1.1 and 1.7 W/g for 15, 30, 45, or 60 min, and their activity was compared to that of a sample heated in a water bath at the same temperature. The residual activity of the exposed samples depends on enzyme concentration, microwave power level, and exposure time; activity was reduced to 10% in 10 μ g/ml solutions treated at 1.7 W/g for 60 min. Microwave effects disappeared at concentrations above 50 μ g/ml. These results were not found following water bath heating at the same temperature and durations. Bioelectromagnetics 20:172–176, 1999. © 1999 Wiley-Liss, Inc.

Key words: thermophilic enzyme; microwave radiation; nonthermal effects

INTRODUCTION

The increasing presence in the environment of electromagnetic fields, from static fields to microwaves, has prompted studies on their effects on biological systems. A large amount of work has been done on this subject over the past decades, and in vivo studies on man and animals, as well as in vitro studies on less complex biological subsystems, have been reported [Osepchuk, 1971; Justesen and Guy, 1977; Justesen and Baird, 1979; Gandhi, 1980; Grandolfo et al., 1981; Toler, 1984; Chiabrera et al., 1985; Polk and Postow, 1986; Franceschetti et al., 1989].

Enzymes, due to their fundamental role in cell metabolism, are probably the simplest system to consider for studying bioelectromagnetic effects in living systems. A limited number of studies related to mesophilic enzymes were carried out in the past [Belkhorde et al., 1974; Ward et al., 1975; Bini et al., 1978; Galvin et al., 1981; Millar et al., 1984; Saffer and Profenno, 1989, 1992], and enzymes isolated from a thermophilic microorganism were recently used [Porcelli et al., 1997].

The action of microwaves (0.3–300 GHz frequency range) on living matter has been widely studied, and most of the observed effects can be explained as being merely due to bulk heating, i.e., temperature increase induced by the electromagnetic field, according to the classical theory of lossy dielectrics [Von Hippel, 1954; Frolich, 1983; Scaife, 1989]. On the other hand, evidence of microwave effects not related to temperature, as measurable by ordinary means, has accumulated over recent years, but the mechanisms involved are still largely unknown due to experimental and modeling difficulties [Saffer and Profenno, 1989, 1992; Stuerga and Gaillard, 1996].

A microorganism that lives at temperatures in the range of 25–45 °C is usually defined as mesophilic. Its enzymes are active up to 50 °C, while at higher temper-

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Received 6 August 1996; Revision received 28 July 1998

atures their activity rapidly decreases. Thermophilic is defined as a microorganism that lives at high temperatures (50–80 °C); its enzymes are thermostable and active up to 80–90 °C.

We used a thermostable enzyme, β -galactosidase from *Bacillus acidocaldarius* (Ba β gal) [La Cara et al., 1992], to assess the effect that enzyme concentration, microwave-specific absorption rate (SAR, W/kg), and exposure duration had on its stability. The key idea is to use sufficient microwave power to increase the enzyme temperature within its thermostable levels and to look for effects that conventional heating would not induce. The experiments were carried out at 10.4 GHz microwave frequency and 70 °C; at this temperature a ± 1 °C variation did not significantly change enzyme activity [La Cara et al., 1992].

MATERIALS AND METHODS

Biological Sample

Experiments were carried out with a β -galactosidase (EC 3.2.1.23) purified from the thermoacidophilic eubacteria *Bacillus acidocaldarius*. The bacterium, isolated from the volcanic area of the Pozzuoli Solfatara near Naples [De Rosa et al., 1973], was grown at 60 °C in a medium containing (per liter): 3 g sucrose, 1 g yeast extract (DIFCO, Detroit, MI), 1 g casaminoacids (DIFCO), 1.3 g (NH₄)₂SO₄, 0.3 g KH₂PO₄, 0.6 g MgSO₄·7H₂O, and 0.1 g CaCl₂·2H₂O. The pH was adjusted to 4.0 with diluted H₂SO₄.

The enzyme was purified according to the procedure described by La Cara et al. [1992] and was employed in homogeneous form (specific activity of 361 U/mg of protein). In all experiments the enzyme was diluted to concentrations between 10–100 μ g/ml in 0.1 M potassium phosphate buffer, pH 6.5. The solution was split into two identical tubes: one was used for microwave exposure, the other was kept in water bath and considered the control. The assay for enzymatic activity was performed immediately after the microwave exposure. An aliquot of 10 μ l of the exposed sample was withdrawn and placed in 1 ml of a reaction mixture containing o-nitrophenol- β -D-galactopyranoside (ONPG) as substrate. The enzyme activity was evaluated following the release of o-nitrophenol as increasing of the absorbance at 405 nm [Pisani et al., 1990]. One unit of β -galactosidase was defined as the amount of enzyme catalyzing the release of 1 μ mole of o-nitrophenol min⁻¹ under standard assay conditions, assuming for the reaction product a molar absorbance coefficient of 3.1×10^3 M⁻¹ cm⁻¹ at 405 nm.

Microwave Exposure

The microwave exposure system (frequency f = 10.4 GHz) is shown in Figure 1a,b. It consists of a WR90

rectangular waveguide system (frequency range 8.2–12.4 GHz, internal dimensions 22.86 mm $\times$ 10.16 mm), in which the power absorbed by the sample (P_a) can be evaluated ($P_a = P_i - P_r - P_t$) by measuring incident, P_i (PM₁), reflected, P_r (PM₂), and transmitted, P_t (PM₃), powers [d'Ambrosio et al., 1995]. A polypropylene test tube containing 0.5 ml of sample was longitudinally inserted into the sample holder (SH). Temperature measurements were carried out during the exposure with two temperature probes (thermocouple Sensortek PT-6) and two temperature monitors (TH, Sensortek TH6D). The leads of the temperature probes were perpendicular to the incident electric field and the small size of the sample gave a slight field perturbation. No changes were observed in temperature reading by turning off the microwave power, and it was assumed that the temperature measurements were not significantly affected by the microwave radiation. Each probe was a point-like (0.029 inches–0.74 mm diameter) thermocouple; they were randomly positioned in the central zone of the sample with a spacing of ~ 5 mm. Differences ΔT between temperature readings were usually 0.5 °C. Maximum observed ΔT was 2.0 °C; this was assumed to be a measure of the maximum temperature inhomogeneity inside the sample. Microwave power adjustments were such that the maximum temperature reading never exceeded 70.5 °C.

A thermally stable environment for the sample was provided. A circulating water jacket guaranteed the prescribed temperature T_0 of the sample holder. The actual sample temperature $T = 70$ °C ± 0.5 °C was higher than T_0 , due to the microwave power P_a absorbed by the sample. During the exposure, some random changes occurred in the power absorbed by the sample, so that in order to keep constant $T = 70$ °C some adjustment of the incident power P_i was provided (variable attenuator VA₁). Every change of the incident, reflected, and transmitted powers was recorded during the exposure, and for each experiment a time average absorbed power per unit mass P_a/m (= SAR, specific absorption rate) was calculated, it being understood that the instantaneous SAR was within $\pm 10\%$ the average value.

The activity of microwave-exposed samples was compared with that of an identical unexposed one (control), which was kept in a water bath at the same constant temperature T of the exposed sample (see below). The prescribed temperature ($T = 70$ °C) was reached within 3 min in both the sample and the control. In order to obtain a microwave heating as fast as the water bath heating, a higher SAR (about double) was used during this period.

All the experiments were performed three times and the average residual activity ($\pm$ SD) is reported. As regards controls, due to the high stability of the thermophilic enzyme at 70 °C and the reproducibility of the

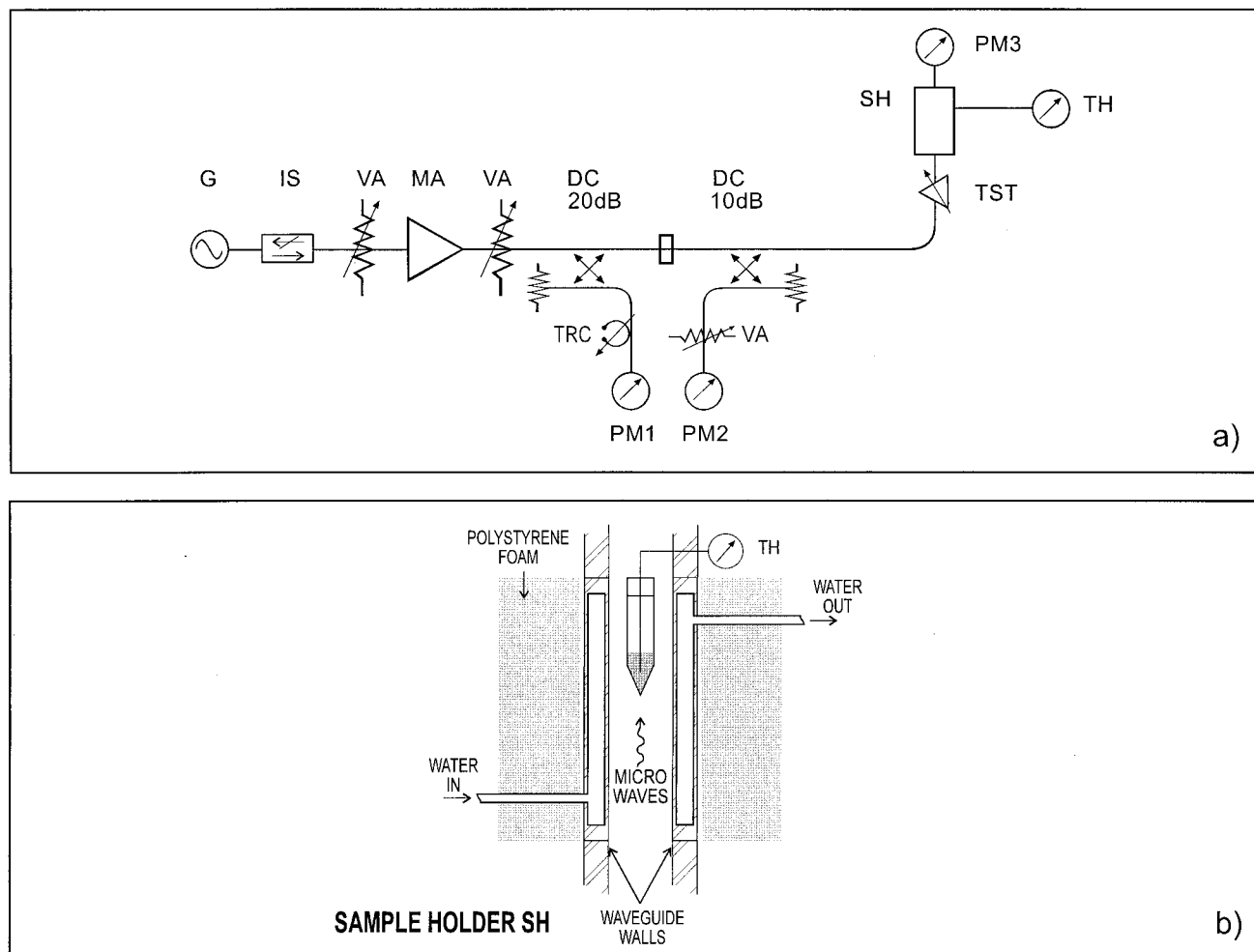


Fig. 1. (a) Microwave exposure setup. G: generator (frequency $f = 10.4$ GHz); IS: isolator; VA: variable attenuator; MA: TWT amplifier; DC: directional coupler; TRC: tunable resonant cavity;

PM: thermocouple and power meter; TST: triple stub tuner; SH: sample holder; TH: thermometer. (b) Sample holder (SH) details.

enzymatic assay, the standard deviation is less than 1%, so no error bars are given.

Experiments

We monitored the enzymatic inactivation: 1) as a function of exposure duration, at a given temperature and SAR. For this purpose, the sample (20 $\mu\text{g/ml}$ protein concentration; 100 mM potassium phosphate buffer, pH 6.5) was kept at 70 °C with microwave SAR level of 1.7 W/g ($T_0 = 20$ °C, $P_i \approx 1.04$ W, $P_r \approx 0.023$ W, $P_t \approx 0.150$ W). A control sample was kept at 70 °C in a water bath. Then the samples were removed from the experimental systems and their residual activity was evaluated and reported as a function of exposure duration Δt ; 2) as a function of enzyme concentration, at given temperature, exposure duration, and SAR. In particular, the tested enzyme concentrations were in the range of 10–100 $\mu\text{g/ml}$. The solutions were ex-

posed at a microwave intensity of 1.1 W/g ($T_0 = 37$ °C, $P_i \approx 0.6$ W, $P_r \approx 0.014$ W, $P_t \approx 0.06$ W) and 1.7 W/g (T_0 and P_i same as in experiment 1). Exposure duration was 1 h. Control samples were kept at 70 °C in water bath for 1 h.

RESULTS AND DISCUSSION

In our first experiment (Fig. 2), samples of Baßgal (20 $\mu\text{g/ml}$) were heated at 70 °C in a water bath (light circles) and with microwaves (SAR = 1.7 W/g, dark circles) for different time durations. Then the samples were removed from the experimental system and their residual activity was evaluated and reported as a function of exposure duration. For each exposure duration the residual activity of three separate microwave-exposed samples was evaluated and the average $\pm$ SD is shown in

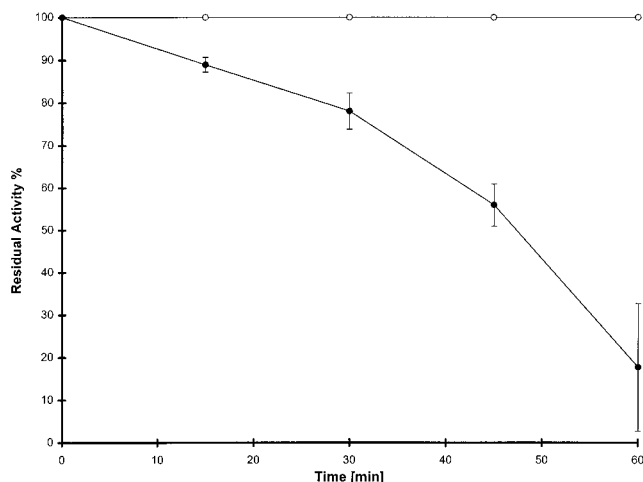


Fig. 2. Enzyme residual activity as a function of exposure duration Δt ($\Delta t = 15, 30, 45, 60$ min). SAR = 1.7 W/g. (○) water bath heated samples; (●) microwave heated samples: average $\pm$ SD.

Figure 2. The effect observed is clear: whereas the activity of the water bath-heated enzyme remained constant even following 1-h heating, the activity of the microwave-heated enzyme decreased as a function of exposure duration. Following a 1-h microwave exposure, the activity was as low as about 20% of the control.

Because many enzymes have been found to be inactivated when diluted below a given protein concentration [Faye and Lambert, 1980], suggesting a sort of instability of the molecule at lower concentration, the microwave effect was also studied as a function of β -galactosidase concentration and microwave intensity at constant exposure duration $\Delta t = 1$ h. As shown in Figure 3, the enzyme inactivation depended on a protein concentration between 10 and 50 μ g/ml and on SAR level (triangles 1.1 W/g, dark circles 1.7 W/g). This effect was not observed at an enzyme concentration higher than 50 μ g/ml.

The enzyme denaturation due to microwaves was irreversible in all cases. It should be noted that after heat treatment an enzyme can often recover its activity at low temperature and in the presence of its substrate. For this reason the microwave-exposed enzyme was incubated at low temperatures (4 and 25 $^{\circ}$ C), with and without its substrate. But in these cases also, no recovery of activity was observed.

The class of thermophilic enzymes is similar to its mesophilic counterpart except for their thermostability [Kristjansson, 1991]. In the literature, significant inactivation of mesophilic enzymes, by 2.45 GHz microwave energy, was reported [Henderson et al., 1975] only for very high values of SAR (62.5–375 W/g). Instead, at 9.375 GHz under intrinsically unstable enzymatic conditions and SAR levels higher than 0.28 W/g, microwaves

were found to increase activity (reduced inactivation) [d'Ambrosio et al., 1989]. Recently, microwave effects that are not due to bulk heating have been observed on thermophilic enzymes; the effects were found to be independent of enzyme concentration [Porcelli et al., 1997]. In the present work, another thermophilic enzyme has been used, and microwave effects not related to temperature, as measurable with ordinary means, have been confirmed; in this case the effects were found dependent on enzyme concentration.

Conventional heating of a protein solution, where the solvent is in high excess, implies an indirect energy transfer from water to the protein caused by collisions. However, microwave heating implies both a direct and indirect energy transfer, because proteins or certain protein domains may exhibit a dipolar moment and resonant oscillations could be induced. These, in turn, may create a mechanical stretching of the proteins due to friction with the medium. This could be an explanation for the inactivation effects following microwave exposure.

As for the role of concentration, it has been confirmed that microwave effect on these molecules is more evident overall when intrinsically unstable enzymatic conditions occur [d'Ambrosio et al., 1989].

CONCLUSIONS

We used a thermostable β -galactosidase isolated from a thermophilic microorganism to study the effects of microwaves on protein stability. We observed that microwave irradiation induced an irreversible inactivation in the enzyme tested that depends on microwave intensity and on protein concentration. At high concen-

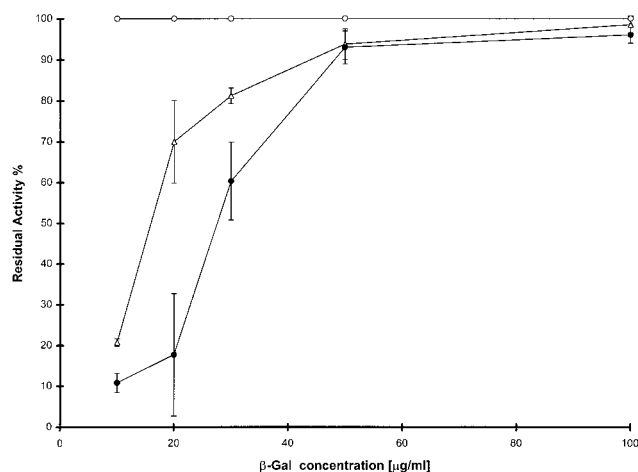


Fig. 3. Residual activity as a function of enzyme concentration. (○) Control samples kept at 70 $^{\circ}$ C in water bath; (Δ) SAR = 1.1 W/g; (●) SAR = 1.7 W/g. Exposure duration ($\Delta t = 60$ min) was the same in all the experiments.

tration this effect disappeared. Inactivation was not found at all following conventional heating.

At this stage, we have no full explanation of the experimental results, but we believe that such biochemical systems could represent a very useful tool for a deeper understanding of the interaction mechanisms between microwaves and biological systems. Possible enzyme conformational changes could be detected by fluorescence and circular dichroism techniques [Porcelli et al., 1997].

Further studies will be performed to establish, at the molecular level, with spectroscopic techniques (such as circular dichroism and/or fluorimetry) the mechanism of enzyme inactivation under microwave exposure.

REFERENCES

- Belkhorde ML, Muc AM, Johnson DL. 1974. Thermal and athermal effects of 2.8 GHz microwaves on three human serum enzymes. *J Microw Power* 9:23–29.
- Bini M, Checcucci A, Ignesi A, Millanta L, Rubino N, Camici G, Manao G, Ramponi G. 1978. Analysis of the effects of microwave energy on enzymatic activity of lactate dehydrogenase (LDH). *J Microw Power* 13:96–99.
- Chiabrera A, Nicolini C, Schwan HP, editors. 1985. *Interactions Between Electromagnetic Fields and Cells*. New York: Plenum Press.
- d'Ambrosio G, Massa R, Gianfreda L, Greco G, Scaglione A. 1989. Influence of microwave radiation on acid phosphatase deactivation process. *Alta Frequenza* 58:361–364.
- d'Ambrosio G, Lioi MB, Massa R, Scarfi MR, Zeni O. 1995. Genotoxic effects of amplitude-modulated microwaves on human lymphocytes exposed in vitro under controlled conditions. *Electro Magnetobiol* 14:157–164.
- De Rosa M, Gambacorta A, Bu' Lock JD. 1973. An isolate of *Bacillus acidocaldarius*, an acidophilic thermophilic with unusual lipids. *Gen Microbiol* 21:145–154.
- Faye L, Lambert C. 1980. The effect of protein concentration on the activity of β -fructosidase from radish seedlings. *Biochim Biophys Acta* 615:392–401.
- Franceschetti G, Gandhi OP, Grandolfo M, editors. 1989. *Electromagnetic Biointeraction: Mechanisms, Safety Standards, Protection Guides*. New York: Plenum Press.
- Frolich H. 1983. *Theory of Dielectrics*. Oxford: Oxford Press.
- Galvin MJ, Parks DL, Mc Ree DI. 1981. Influence of 2.45 GHz microwave radiation on enzymatic activity. *Radiat Environ Biophys* 19:149–156.
- Gandhi OP, editor. 1980. *Proceedings of the IEEE. Special Issue on Biological Effects and Medical Applications of Electromagnetic Energy*, 68.
- Grandolfo M, Michaelson S, Rindi A, editors. 1981. *Biological Effects and Dosimetry of Nonionizing Radiation*. New York: Plenum Press.
- Henderson HM, Mergenroeder K, Stuchly SS. 1975. Effect of 2450 MHz microwave radiation on horseradish peroxidase. *J Microw Power* 10:27–35.
- Justesen RD, Baird RC, editors. 1979. *Radio Science* 14, n. 6S (special issue).
- Justesen RD, Guy AW, editors. 1977. *Radio Science* 12, n. 6S (special issue).
- Kristjansson JK. 1991. Thermophilic organisms as sources of thermostable enzymes. *Trends Biotechnol* 7:349–353.
- La Cara F, Cozzolino R, Nazzaro F, Orlando P, Rossi M. 1992. A novel β -galactosidase activity from thermophilic bacteria *Bacillus acidocaldarius*. Reykjavik, Iceland, presentation at the Int. Conf. Thermophiles: Science and Technology, August, p 32.
- Millar DB, Christopher JP, Hunter J, Yeandle SS. 1984. The effect of exposure of acetylcholinesterase to 2450 MHz microwave radiation. *Bioelectromagnetics* 5:165–172.
- Osepchuk JM, editor. 1971. *IEEE Trans. on Microwave Theory and Techniques, Special Issue on Biological Effects of Microwaves*, 19, n. 2.
- Pisani FM, Rella R, Rozzo C, Raia CA, Nucci R, Gambacorta A, De Rosa M, Rossi M. 1990. Thermostable β -galactosidase from the archaeobacterium *Sulfolobus solfataricus*: Purification and properties. *Eur J Biochem* 187:321–328.
- Polk C, Postow E, editors. 1986. *Handbook of Biological Effects of Electromagnetic Fields*. Boca Raton, FL: CRC Press.
- Porcelli M, Cacciapuoti G, Fusco S, Massa R, d'Ambrosio G, Bertoldo C, De Rosa M, Zappia V. 1997. Non-thermal effects of microwaves on proteins: Thermophilic enzymes as model system. *FEBS Lett* 402: 102–106.
- Saffer JD, Profenno LA. 1989. Sensitive model with which to detect athermal effects of non-ionizing electromagnetic radiation. *Bioelectromagnetics* 10:347–354.
- Saffer JD, Profenno LA. 1992. Microwave-specific heating affects gene expression. *Bioelectromagnetics* 13:75–78.
- Scaife BKP. 1989. *Principles of Dielectrics*. Oxford: Oxford Science Publications.
- Stuerga DAC, Gaillard P. 1996. Microwave athermal effects in chemistry: A myth's autopsy, part 1. *J Microw Power Electromagn Energy* 31:87–100.
- Toler JC, editor. 1984. *IEEE Trans. on Microwave Theory and Techniques, Special Issue on Electromagnetic Wave Interactions with Biological Systems*, 32, n. 8.
- Von Hippel A. 1954. *Dielectrics and Waves*. London: John Wiley & Sons.
- Ward TR, Allis JW, Elder JA. 1975. Measure of enzymatic activity coincident with 2450 MHz microwave exposure. *J Microw Power* 10:316–320.