Effect of Radio Frequency Energy on the Activity of Bowman–Birk Trypsin/Chymotrypsin Inhibitor

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Irreversible and reversible changes in the inhibitory activity of the Bowman–Birk protease inhibitor (BBI) introduced by radio frequency energy (27 MHz) were studied. Inactivation of BBI by means of radio frequency and that by conventional heating with identical time–temperature treatments were found to be similar. No significant extra effect of radio frequency energy on heat inactivation of BBI was demonstrated. The effect of radio frequency energy on the activity of trypsin/chymotrypsin in the presence of BBI at a constant temperature of 25 °C was monitored in situ. Under the influence of the radio frequency field more reaction product was produced, which appeared to result from a change in inhibitory activity. After removal from the radio frequency field, the initial inhibitory activity was recovered, which indicates that the induced effect is reversible in nature.

Keywords: *Electromagnetic energy; microwave; high frequency; enzyme; protein; protease; protease inhibitor; reaction; inactivation; Bowman–Birk inhibitor*

INTRODUCTION

The effects of electromagnetic waves at radio- and microwave frequencies on biological materials have been the subject of many studies. From a human safety point of view substantial attention has been paid to possible health hazards of specific, nonthermal effects of microwave radiation. Currently, there is little evidence for the presence of such a hazard (Foster and Guy, 1986; Roberts et al., 1986; Jauchem and Merritt, 1991). The effect of electromagnetic energy on proteins is of particular interest for many researchers because proteins, e.g., enzymes, are essential for many biological processes. In addition, reduction or enhancement of enzyme activity plays an important role in food processing. In this context, studies were carried out to determine the existence of direct or specific effects of electromagnetic energy, i.e., effects that cannot be explained solely by a rise in temperature of the medium. Belkhode et al. (1974), Henderson et al. (1975), Ward et al. (1975), Yeargers et al. (1975), Allis and Fromme (1979), and Galvin (1981) studied the activity of various enzymes during or after microwave radiation at around 2450 MHz. Overall results showed that in this frequency range thermally induced inactivation took place but no direct "microwave effects" were apparent. At frequencies above 1 GHz the relaxation of water in a material dictates energy dissipation. Takashima (1966) measured the activity of alcohol dehydrogenase before and after irradiation at 1–60 MHz. No change in activity was found after exposure to radiation. Lopez and Baganis (1971) studied the effect of 60 MHz of energy on peroxidase, polyphenolase, pectinesterase, catalase, and α-amylase. Their results indicate that radio frequency energy at 60 MHz does not have any significant effect, aside from heat effects, on inactivation of the five food enzymes studied. However, in these studies activity measurements were carried out subsequent to the radio frequency treatment. In situ measurements of the enzyme activity during the treatment were not included. As also suggested by Grant and Gabriel (1991), at lower frequencies direct interaction of electromagnetic energy with bound water or with (parts of) a macromolecule like a protein is likely to be possible.

In this study we used soybean Bowman–Birk inhibitor (BBI) as a model to study the effects of radio frequency energy on proteins. BBI is a proteinaceous protease inhibitor that inhibits both trypsin and chymotrypsin at kinetically independent binding sites (DiPietro and Liener, 1989). For complex formation with the enzyme, the inhibitor competes with the substrate. However, due to the irreversible character of the enzyme–inhibitor complex, the inhibition has a noncompetitive nature. BBI has a molecular weight of 8000 Da and contains seven disulfide bonds. The reason we focused on BBI as a model protein is its predicted relaxation behavior, availability, practical activity determination in situ, and importance in processing of agricultural materials. Application of an electric field to a aqueous solution of biomolecules leads to relaxation behavior of the Debye type with a rotational relaxation time constant τ estimated from Stoke's law

$$\tau = 4\pi\eta r^3/kT \quad (1)$$

where τ is the rotational relaxation time (s), η is viscosity (Pa·s), r is the radius of the molecule (m), k is the Boltzmann constant, and T is temperature (K).

In this model the dipolar solute molecules are considered spheres, the rotation of which is opposed by the viscosity of the surrounding solvent medium. For BBI the rotation relaxation frequency can thus be calculated to be about 30 MHz. This means that interaction with a radio frequency field of 27 MHz is likely to occur.

This study involved three phases of work. In the first phase we investigated the effects of radio frequency energy on inactivation of the Bowman–Birk inhibitor protein. Experiments were carried out to test our hypothesis that direct coupling of radio frequency energy to the molecule could lead to nonthermal inactivation.

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The purpose of the second part of this study was to examine the effects of radio frequency energy on the activity of BBI in situ (functional effects). In addition, experiments were carried out to determine if functional changes in activity are irreversible or reversible in nature (third part).

MATERIALS AND METHODS

Inactivation by Radio Frequency Energy. To study specific effects of radio frequency energy, conventional heat inactivation of Bowman–Birk inhibitor protein (BBI) was compared with radio frequency heat inactivation. For this reason a solution of 45 mg of Bowman–Birk inhibitor (from soybean; Sigma) in 450 mL of demineralized water (12.5 μM) was heated at 90 °C for 9 h. The radio frequency heating rate was controlled by monitoring and regulation of power input. The radio frequency power input was calculated from the heating rate, heat capacity, and viscosity of the solution and was found to be 168 mW/mL. The sample holder was rectangular with matching electrodes in such a way that the electric field distribution was assumed to be homogeneous throughout the sample. Temperature registration was carried out at four different places in the sample by using four optic sensors (Luxtron). Heating rates were found to be identical at all measured places. The sample was continuously stirred by passing air through the sample holder. Parallel experiments with a conventional heating source were carried out by giving identical time–temperature treatments to the BBI solution, making use of an electrical heating plate. Evaporation was controlled in conventional and radio frequency heating experiments using a condenser with backwash. In both cases 1 mL samples were withdrawn every hour and immediately frozen in liquid nitrogen. The inhibitor activity of these samples toward trypsin/chymotrypsin was analyzed by using the assay of Kakade et al. (1974), as adapted by DiPietro and Liener (1989).

Effects of Radio Frequency Energy on Activity. To study effects on the functional properties of BBI, its inhibitory activity in situ was registered. The effect of radio frequency and energy on the reaction of trypsin and chymotrypsin with their substrate in the presence and in the absence of the Bowman–Birk inhibitor was studied. The rate of product formation (*p*-nitroaniline) from the substrate BAPA (benzoyl-DL-arginine *p*-nitroanilide hydrochloride) and BTPA (benzoyl-L-tyrosine *p*-nitroanilide) was determined spectrophotometrically at 386 nm.

Reaction rates at 25 °C were compared for reaction mixtures placed in the electric field and in a thermostated water bath, respectively.

Reaction Mixture. The total volume of the reaction mixture was 450 mL. Enzyme, substrate, and inhibitor solutions were added to an aqueous Tris buffer solution (0.05 M Tris, 0.02 M $CaCl_2$, pH 8.2). The concentration of BBI was 4.75×10^{-8} M (for trypsin inhibition) and 2.0×10^{-7} M (for chymotrypsin inhibition). This corresponds to an inhibition level of about 50%. The enzyme concentrations were 8.45×10^{-8} M for trypsin and 3.0×10^{-7} M for chymotrypsin. For the trypsin assay we used BAPA at a concentration of 5.78×10^{-4} M. For reaction with chymotrypsin 8.15×10^{-3} M BTPA was used.

Chemicals. Tris(hydroxymethyl)aminomethane, $CaCl_2$, trypsin (from bovine pancreas), chymotrypsin (from bovine pancreas), and BAPA were obtained from Merck; Bowman–Birk inhibitor (from soybean, type V) was obtained from Sigma and BTPA from Boehringer.

Radio Frequency Experiments. The reaction mixture was placed between two electrode plates of a radio frequency unit (Colpitt, The Netherlands; operation frequency 27 MHz). The experimental setup is shown in Figure 1. A volume of 450 mL was irradiated while continuously being cooled by demineralized water of ± 1 °C circulating through a Teflon coil. Demineralized water was used because of its low loss factor at 27 MHz, which prevents coupling of the radio frequency energy to the cooling medium instead of the sample. Liquid mixing throughout the sample was obtained by a bubbling plume. The temperature was monitored with four fiber optical temperature sensors (Luxtron) and found to be the same throughout the solution. Radio frequency power input was calculated to be 270 mW/mL.

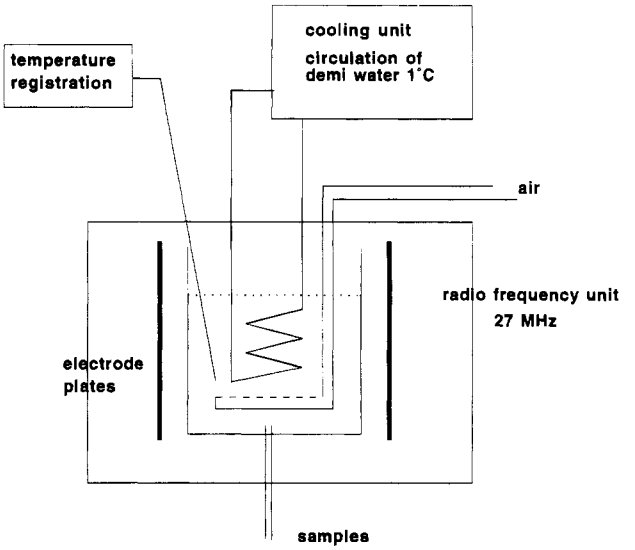

Figure 1. Experimental setup for radio frequency energy input at constant temperature (25 °C).

The effect of radio frequency energy on BBI inhibition of trypsin and chymotrypsin was studied in parallel experiments. Previous research has shown the effect of mixing order of reactants on enzyme-inhibitor activity (Liu and Markakis, 1989; Meijer, 1991). Therefore, in this experiment we used two different procedures for mixing of reactants: S-last and E-last. In the "S-last experiments" enzyme and inhibitor are preincubated and substrate is added last; in the E-last experiments substrate and inhibitor are preincubated and enzyme is added last. The reaction was carried out as follows: 1 mL of BBI solution in demineralized water was added to 354 mL of Tris buffer. In S-last experiments, 4.5 mL of (chymo)trypsin solution in demineralized water was added and the mixture was placed in the radio frequency field. In E-last experiments, 4.5 mL of BAPA or BTPA solution in DMSO was added to the Tris buffer before it was placed in the radio frequency field. Heating rate and cooling rate were brought in balance, and temperature was kept at 25 ± 0.5 °C. After a 5 min incubation period, the last reactant [either BAPA/BTPA (S-last) or (chymo)trypsin (E-last)] was added through a tube into the mixture. The enzyme activity was monitored by measuring absorption at 386 nm of samples taken from solution every 2 min. The reaction was ended after exactly 20 min by addition of 45 mL of acetic acid. The absorption of the total reaction mixture was also measured at 386 nm.

Water Bath Experiments. Apart from the fact that experiments were carried out under thermostated conditions in a water bath, all other reaction conditions were identical to those of the radio frequency assay. Mixtures were kept in the rectangular sample holders in a water bath of 25 °C. Temperature control and stirring were performed as mentioned before.

Control Experiments/Statistical Setup. Both the radio frequency and water bath assay were also carried out in the absence of Bowman–Birk inhibitor (control).

All experiments were carried out in split plot with six experimental units: (1) radio frequency, substrate added last (RF/S-last); (2) water bath, substrate added last (W/S-last); (3) radio frequency, enzyme added last (RF/E-last); (4) water bath, enzyme added last (W/E-last); (5) radio frequency, no inhibitor added (RF/control); (6) water bath, no inhibitor added (W/control). All six experiments were carried out on the same day in random order. Every experimental unit was performed four times. Analysis of variance was carried out to compare the data of reaction rates. Effects were found to be significant if $p < 0.05$.

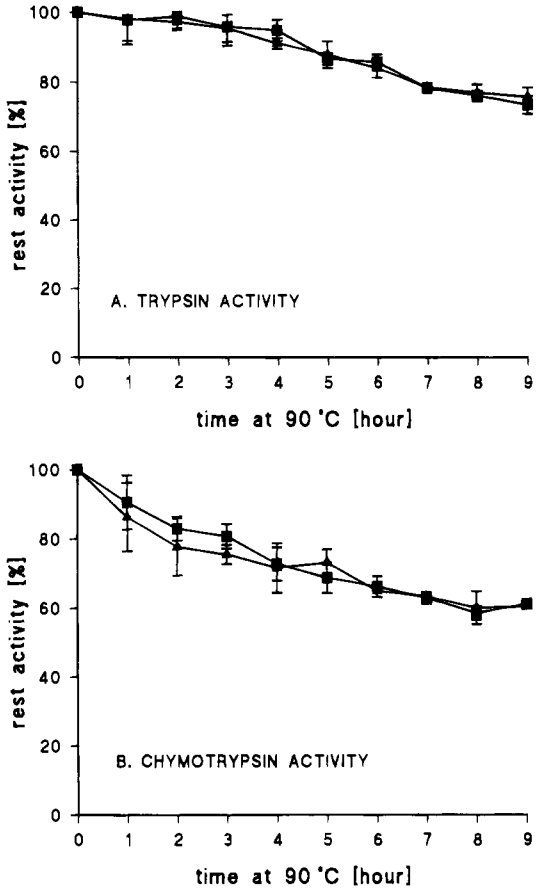

Figure 2. Inactivation of the Bowman–Birk inhibitor by means of conventional heating (■) and radio frequency heating (▲). Trypsin inhibitory activity (A) and chymotrypsin inhibitory activity (B) were measured after heat treatment.

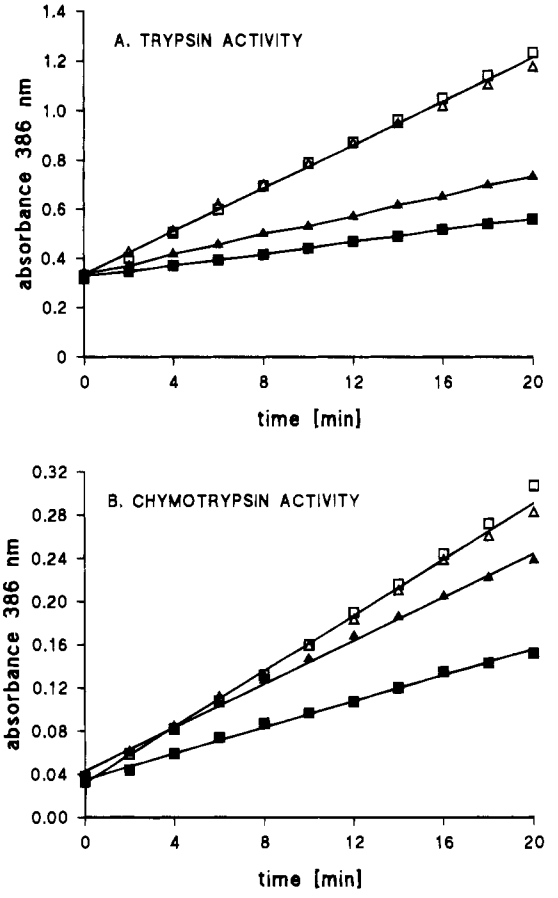

Figure 3. Trypsin activity at 25 °C in the presence of BBI (E-last) [in water bath (■) and in radio frequency field (▲)] and in the absence of BBI (control experiment) [in water bath (□) and in radio frequency field (Δ)]. (B) Chymotrypsin activity at 25 °C in the presence of BBI (E-last) [in water bath (■) and in radio frequency field (▲)] and in the absence of BBI (control experiment) [in water bath (□) and in radio frequency field (Δ)].

Recovery of Activity after Radio Frequency Energy Input. To demonstrate whether radio frequency coupling results in reversible effects on BBI activity, recovery of BBI activity was measured. The same reaction mixture as described under Effects of Radio Frequency Energy on Activity was applied. Inhibition of chymotrypsin activity by BBI was monitored. Radio frequency energy was applied to the reaction mixture which was kept at 25 ± 0.5 °C (also as before). Samples were withdrawn from the reaction mixture every 2 min and read at 386 nm immediately. Every time sample was then placed in a spectrophotometer at 25 °C, and reaction rates were continuously measured for a time period of 20 min.

RESULTS AND DISCUSSION

Inactivation by Radio Frequency Energy. To determine if radio frequency energy has a direct (nonthermal) effect on inactivation of the Bowman–Birk inhibitor, inactivation rates were compared with conventional heat inactivation. Figure 2 shows inactivation curves by means of conventional and radio frequency heating methods for trypsin inhibition (A) and chymotrypsin inhibition (B). Inactivation rates of BBI show no significant differences between the applied heating modes. Inactivation of BBI is very slow (after 9 h at 90 °C there is still 60–85% rest activity). BBI is found to be a very heat stable protein, especially in aqueous solution (DiPietro and Liener, 1989). Chymotrypsin inhibitory activity appears to be more heat sensitive than trypsin inhibition as the total inactivation level is higher. However, there is no significant additional effect of radio frequency energy on inactivation of BBI. Inactivation rates of BBI by conventional and radio frequency energy are similar.

In agreement with the results of Takashima (1966) and Lopez and Baganis (1971), we find that irreversible effects of radio frequency energy on protein conformation, resulting in changes in activity, do not seem to occur. This can be explained by the fact that the photon energy of radio waves is too low (1.12×10^{-7} eV for 27 MHz) to cause bond breaking.

Effects of Radio Frequency Energy on Activity. In Figure 3 typical plots of enzyme activity in the radio frequency field and in a water bath are shown for trypsin (A) and chymotrypsin (B) activity. The linear nature of the reaction over the time period of the assays is shown ($R^2 = 0.99–1.0$). In Table 1 the averages of all runs of trypsin and chymotrypsin activity measurements, calculated from the reaction plots, are given.

Trypsin Activity. No significant change in enzyme activity by radio frequency energy occurs in the control experiments in the absence of BBI (RF/control and C/control). In the presence of BBI an inhibition of trypsin activity occurs, resulting in a decreased formation of the reaction product. The effects of radio frequency and water bath conditions on enzyme activity are compared both for the E-last situation and for the S-last situation.

a. E-last. An increased enzyme activity was found during radio frequency treatment in the reaction when the enzyme was added last (RF/E-last). The reaction rate is about 1.5-fold higher than in the water bath

Table 1. Enzyme Activity (δA_{386}/Minute) for Trypsin and Chymotrypsin, in the Presence or Absence of BBI (Reaction Time 20 min at 25 °C): Effect of Radio Frequency Energy Compared with Reaction in Water Bath and Effect of Mixing Order of Reactants (Substrate Last or Enzyme Last)[a]

	W/control	RF/control	W/S-last	RF/S-last	W/E-last	RF/E-last
trypsin	0.0403[a]	0.0419[a]	0.0119[b]	0.0104[b]	0.0129[b]	0.0190[c]
chymotrypsin	0.0129[a]	0.0132[a]	0.00526[b]	0.00783[c]	0.00593[b]	0.00922[d]

[a] Sample codes: W/control, water bath, no inhibitor added; RF/control, radio frequency, no inhibitor added; W/S-last, water bath, substrate added last; RF/S-last, radio frequency, substrate added last; W/E-last, water bath, enzyme added last; RF/E-last, radio frequency, enzyme added last. Means with different superscripts within rows are significantly different ($p < 0.05$).

experiment. Enhanced formation of the reaction product *p*-nitroaniline points to a reduced inhibitory activity by BBI.

When BBI is free in solution (without enzyme), it may try to align with the alternating electric field. Complex formation between the protease and its inhibitor takes places through a delicate lock–key mechanism. The fact that BBI is moving under the influence of the field may hinder coupling to the enzyme and formation of a complex. This likely leads to reduced trypsin inhibition.

b. S-last. No significant effect of radio frequency energy on trypsin inhibitory activity was found when the substrate was added last (RF/S-last). This can be explained by the fact that in this case a stable complex is formed between enzyme and inhibitor before the reaction mixture is subjected to the electric field. The rate of this complex formation is possibly very high, whereas the rate of dissociation is many times slower, as was demonstrated to occur with other protease inhibitors (Empie and Laskowski, 1982). No equilibrium between association and dissociation of the complex may be reached for the duration of the experiment. In this situation coupling of the electric field cannot hinder complex formation. However, even if we assume this nonequilibrium situation with a very high rate constant for association to take place, we would not expect to find these pronounced differences between the E-last and S-last cases on the applied time scale.

Chymotrypsin Activity. As in the trypsin measurements, no significant change in chymotrypsin activity by radio frequency energy occurs in the control experiments in the absence of BBI (RF/control and C/control).

Chymotrypsin inhibition is influenced by the radio frequency field both when the substrate is added last (RF/S-last) and when the enzyme is added last (RF/E-last). The enzyme activity rate increased about 1.5- and 1.6-fold, respectively.

In the case of chymotrypsin inhibition no great influence of mixing order of reactants was found (also found by M.M.T.M., 1991, unpublished results). It seems that dissociation of the complex of chymotrypsin and BBI occurs at a higher rate than for trypsin and BBI.

In the case of chymotrypsin there is an equilibrium situation where dissociation of the complex leads to free BBI which can couple with radio frequency energy in both mixing sequences.

The results indicate a direct coupling of radio frequency energy with the BBI protein molecules so that BBI is a less ideal substrate for the enzyme. Possibly the electric field causes a shift in the equilibrium between enzyme–inhibitor complex formation and enzyme–substrate complex formation.

Recovery of Activity after Radio Frequency Energy Input. To demonstrate the reversible nature of the radio frequency effect, the recovery of inhibitory activity after exposure to radio frequency energy was measured. Figure 4 shows how at various time points

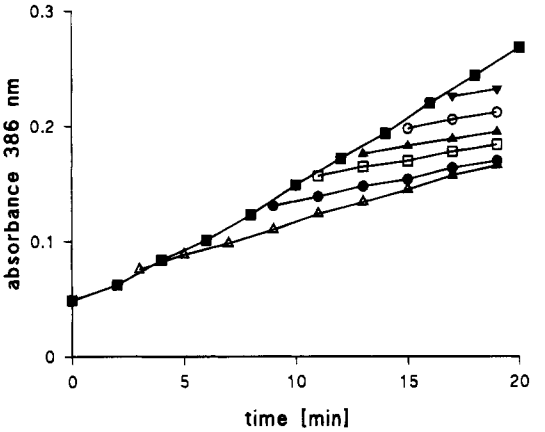

Figure 4. Change in chymotrypsin activity in the presence of BBI (E-last), in and after removal from radio frequency field: (■) reaction under influence of radio frequency energy (25 °C); reaction after removal of sample from radio frequency field at (Δ) 3, (○) 9, (□) 11, (▲) 13, (○) 15, and (▼) 17 min after it was placed in a spectrophotometer at 25 °C.

the enzyme activity rate decreases after removal of the sample from the radio frequency field. This clearly shows that the interaction of radio frequency energy and the inhibitor protein gives rise to an effect that is reversible in nature.

To our knowledge, no other studies were conducted to examine the effects of radio frequency energy on enzymatic activity in situ.

In the area of organic chemistry, however, an increasing number of studies have appeared in which acceleration of reactions as a result of exposure to microwaves is reported (Bose, 1991). A recent finding of Pagnotta et al. (1993) demonstrated a (nonthermal) acceleration of the mutarotation reaction of α-D-glucose in an ethanol/water solvent. The results are not yet confirmed by other researchers. Berlan et al. (1991) presented evidence for a specific activating effect of microwaves on an organic reaction under homogeneous conditions. They found acceleration of several Diels–Alder reactions under microwave conditions at the same bulk temperature as under conventional conditions. To explain these effects, they give two possible mechanisms: a change in entropy resulting in facilitation of the reaction or excitation of rotation of the molecule resulting in activation of molecular collision. In contrast, Raner et al. (1993) were not able to confirm these observations. Jullien et al. (1991) justify the occurrence of nonthermal effects in their theoretical analysis of increase of chemical reactions by microwaves. They present three hypotheses: favorable position because of the electric field, lower entropy state, and increasing probability of molecular collision. They also suggest that differences can occur between high local temperatures of electrical entities. Stuerga et al. (1993) described the possibility of controlling the selectivity of competitive reactions with microwave energy. The isometric ratio in sulfonation of naphthalene could be

influenced by the applied microwave power density. However, this effect is explained by a (controlled) change in heating rate.

In agricultural and food processing research only a few examples of application of specific applications of electromagnetic energy have been published. An example of differential heating is the multifrequency heating of sour milk products (Reuter, 1977). Energy at frequencies of 10–30 and 2450 MHz is used to heat the product mass and the uppermost layer of the product, respectively. However, this heating procedure is not based on any specific coupling effect of the electromagnetic energy with the various compounds in a material. Nelson (1985) describes an application of radio frequency energy to kill insects in grain. Power dissipation at 39 MHz can be 3.5 times higher in the insects than in the grain due to (large) differences in water content and hence in dielectric loss factor. Exposures of 3 s result in 100% mortality in the insects and low temperatures in the grain. Some medical applications of electromagnetic energy for so-called hyperthermia treatments have been reported. These treatments are also based on differences in dielectric properties. Higher conductivity of tumors as compared with normal tissue can result in selective heating of the tumors (Rogers et al., 1983).

In our study we demonstrated a specific effect of a 27 MHz radio frequency field on an enzyme reaction in situ. We also showed that this effect is reversible in nature; i.e., the reaction rate recovers when the solution is removed from the field.

The starting point for this study was to investigate nonthermal effects on BBI by subjecting it to a radio frequency heat treatment. We have shown that inactivation of BBI by radio frequency heating is purely thermal in nature, and from this we can conclude that radio frequency heating could serve as an alternative heating mode for inactivation of proteins (enzymes). Other nonspecific applications, i.e., purely as an alternative heat treatment, of radio frequency energy can be found in the literature. Gluing of wood and plastic welding are examples of very successful nonfood applications, whereas postbaking of biscuits and melting of fat are widespread food applications (Metaxas, 1988). Sunflower seeds and rapeseed were heated successfully with radio frequency energy, leading to higher oil extraction yields and better oil quality. Radio frequency heating appeared to be just as effective as conventional steam toasting in increasing the nutritional value of soybeans (Demeczky, 1985).

In our opinion radio frequency energy forms a good alternative for inactivation of food enzymes in general. The advantages of radio frequency heating such as volumetric, rapid heating, compactness of equipment, and ease of operation offer sound economic alternative heating techniques. However, as investment costs for dielectric heating equipment are still high, the profit on end product quality is often decisive for the economical feasibility of the process.

ABBREVIATIONS USED

BAPA, benzoyl-DL-arginine *p*-nitroanilide hydrochloride; BBI, Bowman–Birk protease inhibitor; BTPA, benzoyl-L-tyrosine *p*-nitroanilide; W, water bath; E, enzyme; RF, radio frequency; S, substrate; Tris, tris(hydroxymethyl)aminomethane.

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