The Effect of Microwave Sublethal Heating on the Ribonucleic Acids of Staphylococcus aureus

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ABSTRACT

The effect of microwave sublethal heat injury on the integrity of S. aureus FRI-100 RNA was examined. The extent of damage to RNA during sublethal microwave heat injury was greater than that occurring as a result of conventional sublethal heating. Although both heating modes resulted in the destruction of the 16S RNA, only those cells heated with microwave energy suffered lesions in their 23S RNA. When cells were allowed to recover, the conventionally heated cells regained normal profiles of the 16S and the 23S RNA after 180 min on the recovery medium. Microwave heated cells restored their 16S RNA after 180 min of recovery. Microwave-injured cells required 270 min to have 23S RNA with sedimentation properties similar to those found in the unheated cells.

The subject of sublethal heat injury and recovery of Streptococcus aureus has been treated by many authors (1,2,4,5,15,16). The loss of salt tolerance is an apparent consequence of heat injury in S. aureus. In fact, the extent of injury is frequently measured by the differential growth on a specific medium and on a non-specific medium. The specific medium often contains 7% sodium chloride. Heat injured S. aureus may also suffer a cytoplasmic membrane damage, cell wall lesions, and loss of intracellular components. The heating menstruum plays a role in determining which of the above mentioned deteriorative actions will take place and to what extent. Among the intracellular components often leaking out of the cell during sublethal heating, 260 nm absorbing materials and Mg$^{2+}$ are commonly detected. Since 260 nm absorbing materials are mainly ribonucleic components and Mg$^{2+}$ is known to stabilize the structure of the ribosomal subunits, the leakage of both components is interrelated. The fact that magnesium plays a role as a protecting agent in the degradation of ribosomes, maintaining the integrity of RNA, has been well documented (5,6,11). Aside from stabilizing its structural configurations, Mg$^{2+}$ also acts as an inhibitor of ribonuclease (11). During heat shock, apparent unwinding of RNA occurs, partially due to Mg$^{2+}$ loss, hence, making it more susceptible to attack by ribonucleases.

Ribonucleases occur in S. aureus and are known to be heat resistant and withstand temperatures much higher than those reached during sublethal heat injury. Many believe that the damage to S. aureus RNA during heat injury is essentially a consequence of enzymatic action facilitated by unfolding of the ribosomal subunits due to heat. Regardless of the specific mechanism governing damage to S. aureus RNA, the fact that it does take place during sublethal heat injury is the focus of this investigation. Specifically, an attempt is made to assess the extent of instability of S. aureus RNA caused by microwave sublethal heat injury. Furthermore, RNA damage due to microwave heating is compared with the ribosomal damage resulting from conventional heat injury. The extent of resynthesis of the ribosomal subunits following recovery from microwave heat injury was also investigated.

Previous results on injury and recovery of S. aureus FRI-100 indicated that the cells suffered greater injury when sublethally heated with microwave energy (9). Even though isothermal conditions were always maintained during microwave heating by cooling the cell suspension during irradiation, the existence of localized overheating within the cell microenvironment is highly possible. The overheating of certain cell components is often referred to as specific thermal effects. Their occurrence is due to variations in the dielectric properties of cell constituents. This will govern the extent of microwave energy that they will absorb, which in turn will determine their heating rate. Another factor that may contribute to localized heating is the water molecules immediately bound to cellular macromolecules. These water shells often possess physical properties different from the bulk water throughout the cell suspension. Accordingly, this water fraction will behave in a different manner in a microwave environment. Depending on the degree of binding, these water molecules may sorb higher fractions of the available microwave energy (8).

These specific thermal effects exist in addition to the ordinary thermal effects resulting from microwave heating and cannot be equalized by cooling conditions (19). Hence, exterior cooling of the cell suspension will not prevent the build-up of temperature gradients and the so-called thermal "spikes" in discrete areas and between particles or layers of different composition.
In this investigation the focus has been on the consequence of specific thermal effects taking place during microwave sublethal heating on the integrity of \textit{S. aureus} RNA.

**MATERIALS AND METHODS**

**Culture preparation**

Frozen stock of \textit{Staphylococcus aureus} FRI-100 was thawed and inoculated into a 6L flask containing 2L of Brain Heart Infusion (BHI, Difco). This culture was incubated at 37°C for 18 h on a rotary shaker. The cells were pelleted by centrifugation at 6000g for 10 min at 4°C. The cell pellets were washed in 0.1M phosphate buffer, pH 7.2. The clean cells were again pelleted by centrifugation and resuspended into 1.5L of the same buffer. The clean cell suspension was divided into three 500 ml portions, a control and two samples for microwave irradiation and conventional heating, respectively.

**Thermal treatments and recovery**

Cells suspended in 0.1M sodium phosphate buffer, pH 7.2, were sublethally heated at 50°C for 30 min using microwave and conventional heating, respectively. Sublethal heat injury was monitored by differential plate counting (9). For the microwave heating part, a modified Sharp carousel microwave oven (Model SKR-7805A, Sharp Electronics Corp., NJ) was utilized. A glass heat exchanger was placed in the oven cavity whereby the cell suspension would flow in a glass coil (0.5 cm i.d. and 1.0 m length) and circulated in a closed loop using a peristaltic pump. The glass coil was fitted within a glass cylindrical tube where kerosene (Fisher Scientific) was allowed to flow in a countercurrent manner. Kerosene was chosen because of its transparency to microwave energy. This arrangement allowed the kerosene to absorb the excess thermal energy from the cell suspension and lose it to a cold water bath at 5°C outside the microwave chamber. By adjusting the sample’s flow rate, that of the kerosene, and the power output of the magnetron, the sample temperature was kept constant at 50°C throughout the 30 min of microwave irradiation. Temperature monitoring was accomplished by inserting two copper-constantan thermocouple leads at the inlet and outlet ports of the circulating sample line, just outside the microwave cavity. Conventional heating was carried out in a hot water bath set at 50°C. Continuous agitation in a wide bottomed flask ensured rapid heating of the cell suspension to 50°C. After heating, the suspensions were cooled to 37°C and each sample was divided into two portions. One portion was prepared for RNA extraction and the other was pelletized by centrifugation. The pellets were subjected to two different physical models for the 30S and the 50S ribosomal subunits. The author stated that four proteins in the 50S subunits were dissolved, large debris was removed by centrifugation at 15,000 g for 20 min.

**Sucrose gradient centrifugation**

Four stock solutions of 5%, 10%, 15%, and 20% sucrose in Tris-chloride buffer, pH 5.0 were prepared. One ml of each was layered carefully in 5 ml ultracentrifuge tubes starting with the heaviest concentration. One ml of the RNA suspension, in the same buffer, was then layered on top of the sucrose gradient. After centrifugation at 32,500 rpm in an SW 50.1 rotor for 24 h at 4°C, the bottom of the centrifuge tubes was punctured with a hypodermic needle and 20 equal fractions were collected. Each fraction was saved for measurement of absorbance at 260 nm (Shimadzu Spectrophotometer, Kyoto, Japan).

**RESULTS AND DISCUSSION**

Among the many possible sites for sublethal heat injury, damage to the cell’s ribosomes is most frequently reported in the case of \textit{S. aureus}. Witter and Ordal (19) reported that the degradation of ribosomes and ribosomal RNA (rRNA) was evident following sublethal stress.

Furthermore, the recovery of injured cells is characterized as a period of rapid RNA synthesis (3). Because of the apparently significant role that ribosomes play in the injury and recovery process, they were examined as a possible site of injury by microwave irradiation. Even though rRNAs seem to be the target of observed lesions, attempts were not made to isolate the rRNA from the tRNA and mRNA. Rather, the total RNAs were examined since rRNA accounts for approximately 80% of the total cellular RNA. Fractionation of the cellular RNA’s for the unheated cells by sucrose gradient centrifugation showed 50S and 30S subunits which correspond to 23S and 16S RNA, respectively (Fig. 1). When the cells were heated by conventional means, selective destruction of the 30S subunits resulted. The 50S subunits were virtually unaffected (Fig. 2). This finding was in agreement with many others reported in the literature, particularly that of Flowers and Martin (4). It is not clear why the 50S subunits are more stable during sublethal heat injury than the 30S subunits. A possible reason may be their structural configuration. Paterakis (12) proposed two different physical models for the 30S and the 50S ribosomal subunits. The author stated that four proteins in the 50S...
subunits, namely the L1, L20, L23, and L24, provide protective regions to RNA against nuclease digestion. These regions apparently failed to exert their protective mechanism on the 50S subunits when microwave energy was utilized for sublethal heat injury (Fig. 3). Similar to conventional heating, microwave heating also resulted in the destruction of the 30S subunits. Degradation of the 50S subunits as well was evident by their shift to approximately 45S. Since the sedimentation coefficient is dependent on the molecular weight and the shape of the macromolecule, a decrease in the Svedberg units indicates a shortening of the nucleotide chain length, simply a loss of proteins and/or subunit unfolding. Microwave heat injury also resulted in a 66% decrease in the 260 nm absorbance corresponding to the 50S subunits as measured by peak height, a possible result of diminishing concentration of the 23S RNA.

In attempting to explain the reasons for the apparently more severe damage to S. aureus RNA induced by microwave sublethal heating, an understanding of the RNA involvement in injury is of importance. It is believed that the breakdown of 23S RNA during sublethal heat injury could be an indirect consequence of heating and, therefore, involves more than nonenzymatic hydrolysis. The temperature used in heat injury may be sufficient to cause some changes in structural integrity of RNA making it more susceptible to attack by ribonuclease (13). Haight and Ordal (5) implicated polynucleotide phosphorylase and ribonuclease for ribosomal degradation of S. aureus MF-31. The thermal stability of micrococal nucleases, in general, and S. aureus nucleases, in particular, is well...
documented. Some purified nucleases are remarkably stable, and only about 14% of their activity is lost during heating for 3 min at 100°C. Therefore, any unfolding of the subunits or other changes caused by heating will most likely enhance enzymatic degradation of RNA. Judging by the more extensive ribosomal damage to the cells heated with microwave energy, it is speculated that microwave heating caused a higher degree of ribosomal subunit unfolding. The protein fractions of the ribosomal subunits are believed to be responsible for the so-called special thermal effects of microwave heating. Proteins in all living cells are surrounded by an aqueous environment which is believed to be the site of the specific action of electromagnetic radiation (14). These specific thermal effects, which essentially are preferentially localized heating, are a result of variations in the relaxation frequency of water, which in turn is governed by the structure of the water molecule (17). Studies have revealed that a certain degree of water binding shifted the relaxation frequency of pure water to approximately 2.45 GHz, which was the frequency utilized in this experiment (8). Similarly, the water sphere surrounding the ribosomal protein may mediate localized heating of the ribosomal subunits, therefore, increasing their unfolding. Pierson et al. (13) indicated that cooperative inter- and intramolecular interactions between the rRNA and ribosomal proteins cause the components to assume specific secondary and tertiary structures. If ribosomal proteins are denatured to a greater extent by microwave heating, then the rRNA structure will lose part or all of its natural arrangement. This may be the factor that facilitates the unfolding and the subsequent exposure of RNA to the available ribonucleases.

Another important factor in the thermal degradation of ribosomes is the availability of divalent cations, specifically Mg²⁺. Some believe that the sublethal heating causes Mg²⁺ loss because of the damage to the cytoplasmic membrane and the cell wall. Hence, ribosomal damage is a consequence of Mg²⁺ loss and is not a direct effect of heat per se (6). It has also been reported that sublethal heating in the presence of magnesium-conserving medium, greatly decreases ribosomal damage. Since the intent of this investigation was to induce ribosomal damage, sodium phosphate buffer was selected as the heating medium. Sodium phosphate buffer chelates approximately 26% of the Mg²⁺ cations from the cells (7). The role of magnesium is essentially to stabilize the ribosomal structure. In the presence of phosphate, the magnesium phosphate complex is slightly soluble, and therefore, it follows that the Mg-dependent ribosomal structure may be damaged. Hurst et al. (7) reported that in S. aureus, heat permits phosphate to reach and react with cellular magnesium. Since microwave heating is suspected of exposing the cellular components to a higher temperature than the bulk suspension, it is likely that phosphate-magnesium interaction occurs to a greater extent.

In order to assess the extent of microwave heat injury to S. aureus RNA, the cells were allowed to recover for various lengths of time, and the ribosomal subunits were re-examined. The cells injured by conventional means required 180 min to attain normal ribosomal subunit sedimentation values. After recovery, the profile of the 30S and 50S subunits was almost exactly the same as the unheated control (Fig. 4). This would indicate an active repair of the damaged ribosomes or synthesis and assembly of new ribosomes. The 180 min of recovery, however, were not enough for total repair from the damage induced by microwave sublethal heating. Although the cells injured by microwave heating regained the normal profile of the 30S (16S RNA) subunits, they did not reach full recovery of the 50S (23S RNA) subunits. Not until after 270 min of recovery, did microwave injured cells show sedimentation properties similar to those of unheated cells.

CONCLUSIONS

Microwave sublethal heating of S. aureus FRI-100 results in greater damage to the cells’ RNA in comparison to conventional sublethal heating. When cells were heated at 50°C for 30 min in Mg²⁺ chelating buffer, the 23S RNA was not affected following conventional heat treatment. On the other hand, microwave heat injury caused destruction of the 23S RNA. When heat injured cells were allowed to recover, it required 180 min of recovery for the conventionally heated cells to regain normal RNA sedimentation values. For the microwave injured cells, 270 min were required to attain RNA sedimentation values similar to those obtained for the unheated cells.

The added injury to cells heated with microwave energy is believed to be due to specific thermal effects that could have subjected the cellular microenvironment to temperatures higher than those reached by the bulk temperature of the suspension. Although other non-thermal effects may be taking place in
addition to the specific thermal effects, it is difficult to prove or disprove their existence. In addition, during microwave heating thermal gradients may exist within the cell depending on its composition and associated differences in dielectric properties.

In summary, it is considered that a better understanding of the mechanisms involved in the microwave inactivation of microorganisms will facilitate the design of more efficient sterilization and pasteurization systems for the food industry.

REFERENCES