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Effects Of Ultrasound On The Digestive Enzyme Pepsin A

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Abstract

Many medical procedures use high frequency sound waves for both treatment and imaging. Even with regular use of ultrasound, there remains an incomplete understanding of its molecular level effects on biological macromolecules. Ultrasound results in acoustic cavitation, the formation, expansion, and violent, implosive collapse of bubbles within a liquid containing dissolved gas. High pressure results in shear-stress and high temperatures (up to 5000°C) at the site of bubble collapse can cause thermally-induced breakdown of the water molecule generating reactive free radical species. Both shear-stress, high temperatures, and free radical reactions, may denaturation nearby biomacromolecules. To more completely understand potential molecular-level effects of ultrasound on biological macromolecules and the operative mechanism of denaturation, the activity of the digestive enzyme pepsin (a 35 kDa protein with primarily beta-sheet secondary structure) was measured as a function of sonication time using a stop-point assay of hemoglobin degradation. After a one-hour sonication, enzyme activities typically decreased by ~40%, suggesting a sonochemically-induced structural change to the enzyme active site. To assess whether free radicals were responsible for loss of enzyme activity, pepsin was sonicated in the presence of the free radical scavenger, n-butanol, up to 0.1 M. Enzyme activity decreases was significantly less (<5%) in the presence of scavenger which showed that radical reactions that alter the enzyme structure are the principle mechanism for enzyme denaturation. The compact shape and relatively small size of pepsin likely protects the enzyme from significant shear-stress.

Keywords: sonochemistry, sonication, ultrasound, pepsin, free radicals

1. Introduction

Despite the extensive use of ultrasound in medicine, a comprehensive understanding of the effects of ultrasound on biomacromolecules, including enzymes, in cells and tissues in treatment areas remain elusive. Changes to the *in vivo* environment and external forces can alter protein three-dimensional structure resulting in a loss of or change in function. Thermal effects, shear-stress, and free radical reactions are possible mechanisms by which sonication may alter inter- and intramolecular covalent and noncovalent interactions.[1-3]

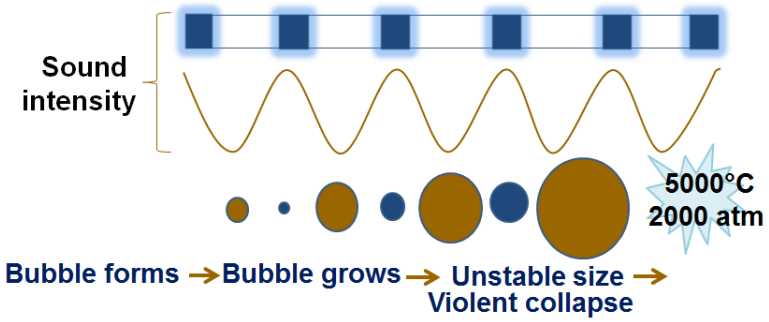

Figure 1. Concentration of hydroxyl radical as a function of sonication time. Inset shows the absorption spectra of Fricke solution after 12 min sonication.

Sonochemistry results from ultrasonically-induced acoustic cavitation (Figure 1), which is the formation, expansion, and violent, implosive collapse of bubbles within a liquid containing dissolved gas.[4-7] At the cavitation surface, high pressure (up to 2000 atm) results in high shear-stress. High temperatures (up to 5000 °C) at the site of bubble collapse can cause the thermally-induced homolysis (breakdown of a neutral molecule generating free radical species) of water resulting in the formation of radical species (Equation 1). Free radicals, in particular reactive oxygen species such as hydroxyl radicals (•OH), are often associated with cell damage that may results in disease states.

$$H_2O \rightarrow H^{\bullet} + {}^{\bullet}OH \qquad (1)$$

Irradiating aqueous protein solutions with high-energy gamma rays generates hydroxyl and protein radicals in aqueous media.[8] Several radical reactions occur in irradiated aqueous solutions; namely, fragmentation, the breaking of a small number of peptide bonds to form polypeptides of shorter length than the original protein, and multimerization, or cross-linking, of individual protein molecules. Comparable radical reactions may be expected when aqueous protein solutions are exposed to ultrasound.

For this study, the well-characterized digestive enzyme pepsin A was used. This digestive enzyme (36 kDa, 327 amino acids) is active between *p*H 1-5 and degrades food proteins into peptides. The enzyme cleaves at the carboxyl (–COOH) group of aromatic amino acids. Pepsin A is a"β-protein" complex due to the tertiary hydrogen bonding between β-sheets. The structure of the enzyme active sight is large and crescent-shaped.

Previously, Chambers[9] showed that exposing pepsin to ultrasound resulted in its deactivation. However, Chamber's work, and those on other enzyme systems, have not assessed whether enzyme activity changes result from the action of free-radicals and/or hydrodynamic shear from the implosive collapse of bubbles.

This study aims to assess the molecular-level effects of ultrasound on enzyme structure and activity before and after exposure to ultrasound in aqueous solutions. Furthermore, the operative mechanism of denaturation will be elucidated; namely, are reactions with radicals and/or hydrodynamic shear stress resulting from acoustic cavitation are the predominant cause of enzyme activity changes.

2. Methodology

2.1 Chemicals

All chemicals for this work were of highest purity and were use as received from Sigma-Aldrich Company.

2.2 Sonication

Samples to be sonicated (aqueous pepsin and Fricke solutions) were placed into a 25 × 150 mm Pyrex test tube centered in a Cole-Palmer ultrasonic cleaner (Model B3-R; frequency = 55 kHz; nominal power = 17 W).

2.3 Fricke Dosimetry

A 3-mL aliquot of ferrous-sulfate (Fricke) solution (0.001 M $Fe(NH_4)_2(SO_4)_2$; 0.8 N H_2SO_4; 0.001 *N* NaCl) was sonicated for a given amount of time. Optical absorption spectra were obtained using a Lamda 25 UV-Vis spectrophotometer (Perkin-Elmer). Absorbance at 305 nm and the extinction coefficient (2090 $M^{-1}cm^{-1}$) were used to determine the ferric ion, Fe^{3+}, concentration.[10]

2.4 Stop-point (rate) assay

Enzyme activity was measured using a stop-point assay according to Anson.[11] Purified pepsin A (0.5 mg/mL) (Macron Fine Chemicals) in 0.01 N HCl solution was added to hemoglobin substrate and incubated at 37°C for exactly 10 minutes. The hydrolysis of denatured hemoglobin (in 0.3 M HCl) was stopped by adding 5% trichloroacetic acid (TCA). For blanks, enzyme was added after TCA. Samples were filtered and filtrate absorbance measured at 280 nm using the aforementioned spectrophotometer. To calculate the percent activity, the ratio of filtrate absorbance of sonicated samples to filtrate absorbance of unsonicated samples was calculated.

2.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Analysis of pepsin samples were done using SDS-PAGE according to Laemmli.[12] Samples were boiled for 2 min at 100°C in sample buffer. SDS-PAGE was done using a Bio-Rad Mini-Protein Tetra Cell System (model 165-8001) and EC250-90 power supply (E-C Apparatus Corporation). Proteins were visualized by Coomassie Blue staining.

3. Data

3.1 Characterization of the Ultrasonic Bath and Fricke Dosimetry

Fricke Dosimetry was used to (1) confirm that acoustic cavitation occurred in the ultrasonic bath and (2) to quantify the concentration of radicals formed per unit time. The optical absorption in the wavelength range of 200 to 350 nm after sonication of an aerated solution of 1 mM ammonium iron (II) sulfate hexahydrate in 0.8 N sulfuric acid was monitored. The change in the optical adsorption of this solution after sonication for a given amount of time indicated that hydroxyl radicals formed via the thermally-induced homolysis of water caused by acoustic cavitation. The hydroxyl radicals generated oxidized ferrous ion to ferric ions, which absorb maximally at 305 nm. Using the Beer-Lambert law, the concentration of ferric ion at each sonication time was determined, as shown in Figure 1, and increased linearly as a function of sonication time. Approximately 10 μM of ferric ion were generated per minute of sonication, which corresponds to a concentration of 10 μM hydroxyl radicals (the hydrogen radicals are converted to hydroxyl radicals via reactions with water molecules).

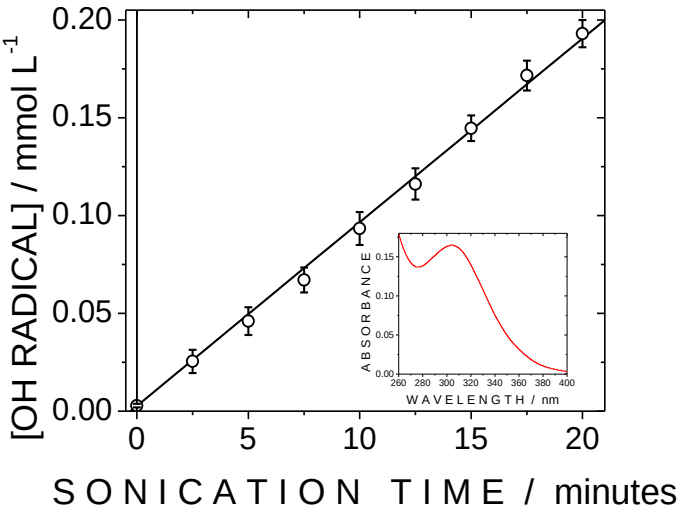

Figure 2. Concentration of hydroxyl radical as a function of sonication time. Inset shows the absorption spectra of Fricke solution after 12 min sonication.

3.2 Sonication of pepsin A

Aqueous pepsin A solutions were sonicated from 0 to 60 minutes in the aforementioned ultrasonic bath. Analysis of stop-point assay results showed that pepsin A activity decreased by ~40% after a 60 minute sonication (Figure 3, ○). This decrease in enzyme activity suggests structural changes occurred that affected the enzyme active site.

3.3 Sonication of pepsin A with radical scavenger *n*-butanol

To assess the potential role of free radical in the mechanism of denaturation, aqueous pepsin A samples were sonicated in the presence of a radical scavenger n-butanol. N-butanol is a suitable radical scavenger because its boiling point is similar to water, is soluble in water, and expected not to affect the temperature inside the bubble (which depends on the vapor pressure).[13] Hydroxyl radicals can abstract hydrogen atoms from n-butanol to generate water and diols according to Equation 2.[14]

$$CH_3(CH_2)_3OH \xrightarrow{\bullet OH} \bullet CH_2(CH_2)_3OH + H_2O \xrightarrow{2\times \bullet R-OH} HO(CH_2)_8OH \qquad (2)$$

The effect of n-butanol on native enzyme activity was evaluated by sonicating pepsin with various concentrations of n-butanol and measuring activity using the stop-point assay. It was determined that n-butanol concentrations up to 0.10 *M* did not affect the native enzyme activity.

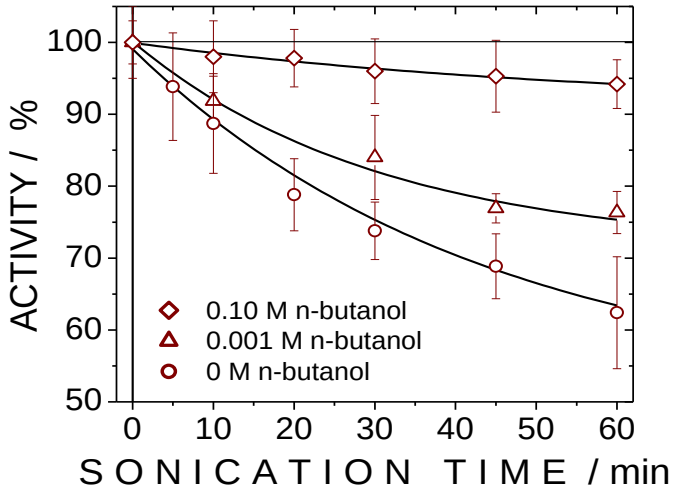

Figure 3. The effect of sonication on pepsin activity with (◇, △) and without radical scavenger (○). Fits are shown to guide the eye.

Sonication of pepsin A in the presence of radical scavenger resulted in enzyme activities that were higher at all sonication times (Figure 3, △,◇). For instance, after a 60-minute sonication with 0.001 *M* n-butanol present, enzyme activity decreased by 25%, and with 0.10 *M* n-butanol, the activity decreased by only five percent. This suggests that the radical scavenger n-butanol protected the enzyme from free radicals reactions that may alter the enzyme structure.

3.4 Biochemical assay

SDS-PAGE was used to assess size changes and, in turn, structural changes that may have resulted in activity changes in sonicated enzyme samples. Free radical reactions can result in intramolecular free radical reactions and/or intermolecular free radical reactions (Equations 3 and 4). In the case of intermolecular radical reactions, protein multimers (i.e., dimers, trimers, etc.) having higher molar masses (sizes) than monomeric species are expected. Intramolecular protein radical reactions will not result in a molar mass change. There is also the potential that enzyme primary structure may be disrupted by radical reactions or shear-stress that smaller molar mass fragments may result be detected.

$$\text{Protein}\bullet + \text{Protein}\bullet \rightarrow \text{Protein—Protein} \qquad (3)$$

$$\text{Protein}\bullet \rightarrow \text{Protein}' \qquad (4)$$

Figure 4 shows the results of the SDS-PAGE analysis before and after a 60-minute sonication. In the presence and absence of radical scavenger, pepsin multimers (molar mass > 35 kDa) and fragments (molar mass < 35 kDa) were not observed. The absence of a molar mass change suggests that intramolecular protein radical reactions dominate. Also, the nonappearance of fragments indicates that protein primary structure structure was conserved.

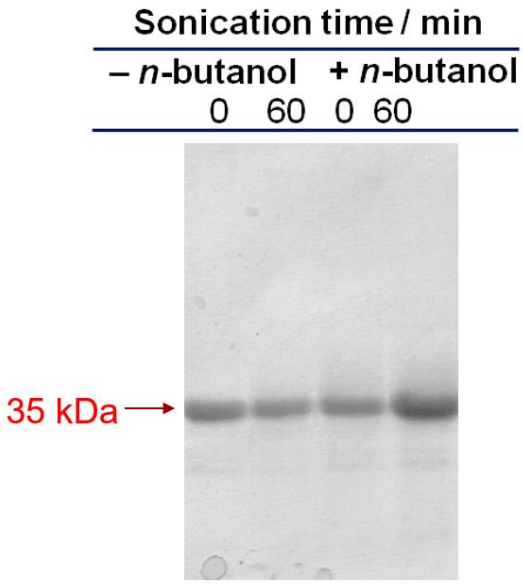

Figure 4. Representative SDS-PAGE of pepsin A with and without scavenger before & after a 60-minute sonication.

4. Conclusion

SDS-PAGE analysis showed that protein multimers and fragments were not detected indicating that intramolecular protein radical reactions dominate. Enzyme activity decreased less in the presence of radical scavenger, further establishing that intramolecular protein radical reactions that change protein structure are the principle mechanism for enzyme denaturation. The compact shape and small size of pepsin protects the enzyme from the effects of hydrodynamic shear.

5. Future Work

Further assessment the effect of ultrasound on enzyme secondary structure will be made by performing spectropolarimetry experiments. In the long-term, crystallography of sonicated pepsin may provide a clearer picture as to why exposure to ultrasound causes a decrease in enzyme activity; namely, how is the active site changed. This work will also be expanded to look at how ultrasound affects the activity of digestive enzymes with different principal primary structures.

6. Acknowledgement

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