THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF CHEMISTRY

IRREVERSIBLE INACTIVATION OF PORCINE PEPSIN BY Ɣ-IRRADIATION IN AQUEOUS SOLUTION

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ABSTRACT

The effects of Y-irradiation on the activity and structure of pepsin from porcine gastric mucosa were studied. 1% w/v aqueous enzyme solutions were irradiated with doses of 1, 2.5, 5, and 10 kGy; pristine enzyme was used as a control for normal enzyme behavior. Purified, lyophilized ovalbumin from *Gallus gallus* eggs was used as the substrate for proteolytic cleavage. The activity of pepsin was measured by the appearance of soluble cleavage products determined by a spectrophotometric assay for primary amines; measurements were compared to standard curves created using pure leucine and valine solutions. Nominal changes in the structure of the enzyme were analyzed using sodium dodecyl sulfate gel electrophoresis. Measurement of product formation during the time-resolved digestions of ovalbumin performed with the irradiated enzyme solutions indicated a positive correlation between loss of enzyme activity and increasingly greater doses of radiation. The radical reactions and scission induced by Y-radiation, which have been studied in previous experiments, appear to have the ability to irreversibly inactivate pepsin. These results underscore the dependence of protein function on structure, and immediately suggest several different ways through which radical reactions may inactivate an enzyme's catalytic ability. Further studies following up on these results could yield a better understanding of the exact nature of how radiation affects more complicated *in vivo* systems.

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Introduction

Radiation-induced damage of proteins is an important concern in the context of biological systems as well as from a physicochemical standpoint. The function of a protein is intrinsically related to its overall structure; from primary sequence to quaternary structure, proteins derive their staggering versatility from the many different conformations they can fold into. It has been well-established that changes in environmental variables such as temperature and pH can disrupt the folding of a protein and therefore its function. Radiation has also been shown to alter the structure of proteins, but the underlying chemical phenomena behind the effects of radiation on proteins have only recently begun to be described. The effects of radiation differ from those of temperature and pH in that structural changes entail the alteration of the covalent bonds in the protein. While hardy proteins may be able to refold into their correct conformations, alone or with the help of chaperones, covalent changes cannot be reversed.

The effects of radiation on proteins in solution may be categorized into direct effects and indirect effects. Direct effects are a result of the absorption of Y-radiation by the protein molecules themselves. Indirect effects result from interactions with other species that were generated by radiolysis. In aqueous solutions, indirect effects dominate since the system is mostly water. The majority of the energy given off by a radiation source will be absorbed by the water, generating radiolytic products which will then react with the protein in solution.

Analyses of the radiolytic products of amino acids, low molecular weight peptides, and proteins revealed three possible ways through which Y-irradiation can affect structure: Intramolecular radical reactions resulting in the formation of intramolecular bonds, intermolecular reactions leading to agglomeration lone molecules, and radiolytic scission of

existing bonds, resulting in fragmentation[5]. These reactions are catalyzed by the formation of aggressive radical species in solution, particularly OH radical. Formation of the OH radical starts with ionization of water. A series of reactions occurs, eventually leading to the formation of OH radical, hydrated electron, and H.

$$H_2O \rightarrow H_2O^+ + e^-_{aq} \rightarrow \dot{O}H + e^-_{aq} + \dot{H}$$

G values are defined as the number of events occurring for every 100 eV of energy absorbed. For OH radical, hydrated electron, and hydrogen, the values are 2.7, 2.7, and 0.5, respectively[2]. A system that has both OH radical and hydrated electron is simultaneously oxidizing and reducing; such a system is complex compared to those that are either oxidizing or reducing. If irradiation is carried out in the presence of N_2O, the hydrated electron can be converted into OH radical, effectively doubling the G value of OH radical and creating a completely oxidizing environment. This conversion proceeds as follows:

$$N_2O + e^-_{aq} \rightarrow N_2 + O^-$$
$$O^- + H_2O \rightarrow \dot{O}H + OH^-$$

OH radical typically abstracts hydrogen from a protein, producing water and a protein radical that can now either react with itself or other proteins in solution.

$$\dot{O}H + \text{Protein A} \rightarrow H_2O + \text{Prot}\dot{\text{e}}\text{in A}$$

Possible subsequent reactions include propagation of the radical reaction and termination by the combination of two existing radicals. Detailed mechanisms of preferential reactions have been described[2]. In the case of globular proteins, surface residues are most likely to react with the OH radical. Interior residues are not susceptible to radical attack as the OH radical cannot diffuse into the interior of the protein. As shown in the crystal structure of pepsin[7] in Figure 1, the

enzyme being irradiated in this study is a globular protein, so it can be expected that most radiation chemistry will occur at the surface residues of the protein.

Figure 1: Porcine pepsin crystal structure. As a globular protein, it's surface is mostly hydrophilic residues while hydrophobic residues are buried in the interior.

This knowledge has provided the foundation for more systematic studies of the effects of radiation on proteins. Subsequent experiments have used aqueous solutions of ovalbumin from *Gallus gallus* eggs as a model system for preliminary work[3, 8, 9]. This system offers some advantages: It is inexpensive, easy to produce, and compared to in vivo systems, relatively simple. Yet the knowledge gained from such a simple system has allowed some general conclusions to be made about the behavior of more complex protein systems. For example, dose-dependent and concentration-dependent studies indicate that at relatively low dose rates of γ-radiation, the agglomeration reactions of proteins proceed primarily by grafting instead of cross-linking[6]. That is, agglomeration occurs by the reaction of a protein radical with an intact molecule rather than the recombination of two protein radicals. There are not enough radicals in solution

$$\dot{\text{Protein}} + \dot{\text{Protein}} \rightarrow \text{Dimer} \quad \text{(Cross-Linking)}$$

$$\dot{\text{Protein}} + \text{Protein} \rightarrow \dot{\text{Dimer}} \quad \text{(Grafting)}$$

for the combination of two radicals to be a likely event; instead, radicals more often react with pristine molecules. This is reflected in the independence of the G value of ovalbumin agglomeration in aqeuous solution within the dose rate range 0.001 – 0.1 Gy/s[8]; it does not matter how many OH radicals are produced at once as long as some are actually produced to start the series of radical reactions. Since the grafting process creates novel molecules not found in native ovalbumin, changes in the physical properties of the solution can also be anticipated; a semiemprical equation has been developed relating the viscosity of solution to the dose of radiation[3]. Of course, more work needs to be done to fully characterize the behavior of this system.

Anything that affects the form of a protein can also affect its function. Enzymes present a unique opportunity to study the effects of radiation-induced structural changes in proteins since their proper, native structure is reflected in their ability to catalyze biochemical reactions. Therefore, any changes in the activity of a particular enzyme then may be attributed to the gross changes to the enzyme's three-dimensional structure.

While past studies have been done on the effects of radiation on enzyme activity, they were done so when knowledge of the underlying chemistry was less well-understood[8]. The elucidation of the mechanisms of the radical reactions in aqueous protein solutions[2, 6] now provide a better context for more in-depth study of this phenomenon. Also, the advent of a new biochemical methods, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), makes it possible to some degree to follow the effects of radiation on the overall structure of a protein and connect those changes to any differences in activity. It is therefore worthwhile to revisit the effects of irradiation on enzymes with the new knowledge and experimental techniques available. This paper describes the effects of irradiation on the activity and structure of pepsin from porcine gastric mucosa under anaerobic, radical-producing conditions.

Materials and Methods

Preparation and Irradiation of Pepsin Samples

A 10 mg/mL pepsin solution was prepared in dH_2O using commercially available lyophilized powder (Pepsin from porcine gastric mucosa, Sigma P7012, 2500-3500 IU/mg protein). Aliquots were dispensed into glass tubes and repeatedly purged with N_2O; after each purge, the glass tubes were sealed with rubber septums and gently inverted to ensure good contact of the solution with the N_2O. This was done six times for each tube, and each purge lasted 9 minutes. After purging, the glass tubes were sealed with a propane torch to ensure that no oxygen would leak in prior to irradiation. Samples were irradiated at the Breazeale Nuclear Reactor with a Co^{60} γ-ray source at a dose rate of 6.7 kGy/hour; they were removed from the source at different times such that pepsin samples were obtained with radiation doses of 2.5, 5, and 10 kGy.

Pepsin Activity Assay

The activity of pepsin was confirmed using a variation of the assay described by Anson[1]. A 2.0% w/v hemoglobin solution was prepared with a 60 mM HCl concentration. 5 mL of this solution was equilibrated at 37°C and combined with 1 mL of a test pepsin solution (0.04 mg/mL) prepared in 10 mM HCl. The mixture was incubated in a water bath for 10 minutes at 37°C before the addition of 10 mL of tricholoroacetic acid (5% w/v). For the blank, enzyme was added after the addition of TCA. Upon addition of TCA, insoluble hemoglobin molecules and fragments precipitated out of solution; the activity of pepsin was determined by the presence of TCA-soluble peptides. The resulting suspension was swirled and incubated for another 5 minutes

before being filtered through Whatman filter paper. The filtrates were each transferred to a quartz cuvette, and their absorption spectra measured in a Varian Cary 4000 UV-Vis Spectrophotometer. The difference in absorption at 280 nm was measured to confirm the activity of the enzyme.

Ovalbumin Digestion: Time-Resolved Studies

Ovalbumin samples were digested with pepsin in a solution with pH 1.8 at 37º C. Time-resolved digestion mixtures consisted of 3 mL of ovalbumin solution (10 mg/mL) in sodium phosphate buffer (0.1 M, pH 7.4), 0.06 mL HCl (6 M), and 0.75 mL pepsin (0.4% w/v). Separate mixtures were made for each of the irradiated pepsin solutions. The components of the digestion were combined and incubated in an isothermal water bath. Upon addition of pepsin to the digestion mixtures, aliquots were removed at 5, 10, 20, 30, and 40 minutes. Control mixtures that contained 0.06 mL dH_2O instead of HCl were used for samples taken at time 0. Collected aliquots were immediately diluted 10-fold in $NaHCO_3$ solution in order to raise the pH of the reaction and stop the activity of pepsin. These aliquots were then assayed for peptide concentration.

Ovalbumin Digestion: Substrate-Dependent Studies

Digestions with varying substrate concentrations were performed to determine the kinetic parameters of pepsin in its irradiated states. These digestions were also carried out at pH 1.8 and 37° C. The mixtures for these digestions were scaled-down versions of the recipe used for the time-resolved reactions, consisting of 0.5 mL ovalbumin (2.5, 5, 7.5, 30, and 50 mg/mL), 0.015 mL HCl (6M), and 0.125 mL pepsin (0.4% w/v) irradiated at various doses. Separate mixtures containing the same components except for the substitution of HCl with DI water served as undigested samples at time 0. The mixtures were incubated for 5 minutes before being diluted 10-fold in $NaHCO_3$. These 10-fold dilutions were also assayed for peptide concentration.

Peptide Concentration Assay

The peptide concentrations of the aliquots taken from the digestion mixtures were determined by the method described by Sataki, et al[6]. The assay was performed by combining 1 mL protein sample, 1 mL 2,4,6-trinitrobenzylsulfonic acid (0.1% w/v), and 1 mL $NaHCO_3$ (4% w/v); the final pH was 9. For the blank solution, 1 mL dH_2O was used instead of protein sample. The solutions were shielded from ambient light and incubated in a heating block at 40ºC for 2 hours. 1 mL HCl (1 M) was added to each solution to acidify them, stabilizing the TNP-peptide derivatives. Each solution was then centrifuged, bringing down any precipitated protein into a pellet. The supernatant was transferred to a 4 mL acrylic cuvette, and the absorbance at 340 nm was measured. Peptide concentrations were determined using standard curves generated with DL-valine (Sigma 94640) and D-leucine (Sigma 855448) solutions of known concentration.

SDS-PAGE

Aliquots taken from the pepsin solutions were subjected to SDS-PAGE according to the method described by Laemmli. Electrophoresis was performed using a Bio-Rad Mini-PROTEAN Tetra Cell and a Bio-Rad PowerPac Basic power supply. 10% separating gels were used to be consistent with procedures from the previous experiments on irradiated aqueous ovalbumin (3, 7, 6). Stacking gels were 4.4% polyacrylamide. Protein samples with concentrations of 15 μg/mL were used. Gels were run at 100V for 2 hours, then stained with a 0.1% Coomassie Blue solution in 50% TCA. After staining, the gels were destained with a 7% acetic acid solution.

Results

Before any experiments could be performed, the lyophilized pepsin used throughout the experimental procedures had to be positively assayed for activity. Figure 2 shows the absorption spectra of the test and blank solutions from the pepsin activity assay. The appearance of a peak at 280 nm in the test solution indicates the presence of TCA-soluble peptides produced from the proteolytic cleavage of hemoglobin. The pepsin was therefore active.

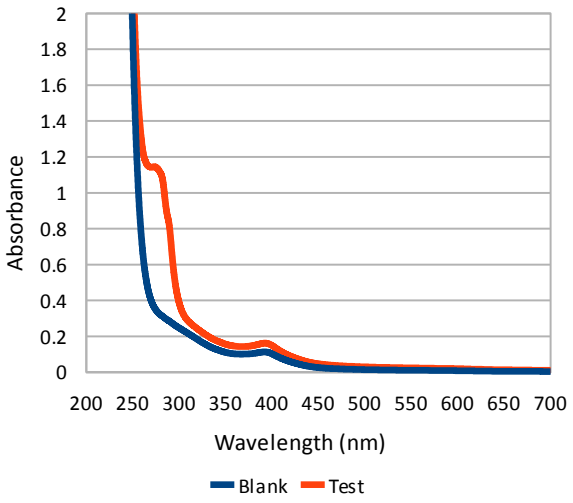

Figure 2: Absorption spectra of blank and test solutions from pepsin activity assay.

Figure 3 shows the absorption spectra of a blank solution and a digest sample after being assayed for soluble peptide. The greater absorbance at 340 nm for the digested sample is a result of the formation of TNP-peptide derivatives. When TNBS reacts with a primary amine to form a TNP-amino derivative, there is a proportional increase in the peak absorbance at 340 nm. Since proteolytic cleavage liberates primary amines, this assay can be used to measure the extent of proteolysis of ovalbumin by pepsin.

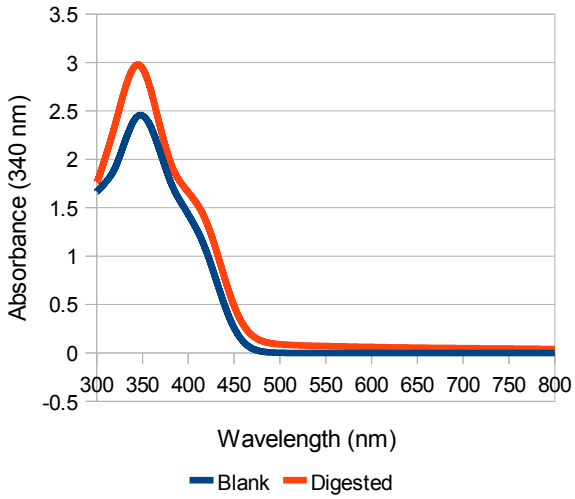

Figure 3: Absorption spectra of solutions from spectrophotometric assay for peptides.

Figure 4 shows the standard curves used for the spectrophotometric assay of primary amines. As demonstrated by Satake, et al, these standard curves showed that A340 was proportional to concentration in the range of 0.01-0.8 µmol/mL. The equations of these standard curves are nearly identical, demonstrating the similarity of the extinction coefficients of different

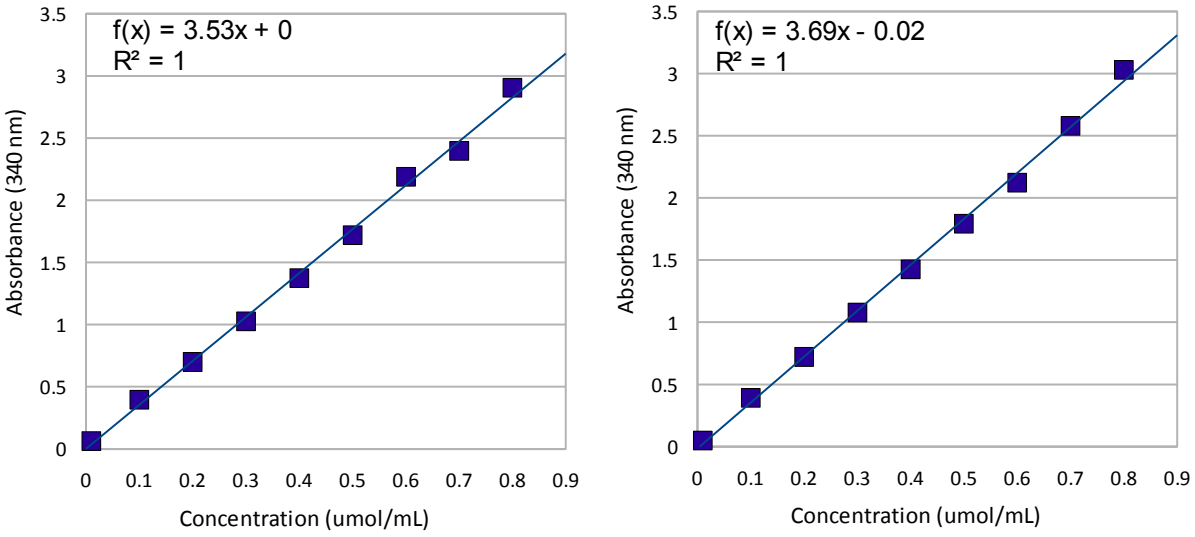

Figure 4: Standard curves for the spectrophotometric assay of primary amines. The one on the left was generated using Leucine, and the one right with Valine.

trinitrophenyl (TNP) derivatives. Since the extinction coefficients of TNP derivatives are practically the same for any primary amine whether it be an amino acid, a peptide, or a protein, the spectrophotometric assay with TNBS is useful for quantifying the peptide content in a heterogeneous solution.

This assay was used to measure the change in peptide content in solution due to the action of pepsin on ovalbumin. Figure 5 superimposes the data points obtained from assays of digestion mixtures over a period of 40 minutes. Although the reaction mixtures started with similar absorbances, digestions performed with irradiated pepsin appear to evolve less color over time than those carried out with native or lower-dose irradiated enzyme. This is already an indicator of activity loss.

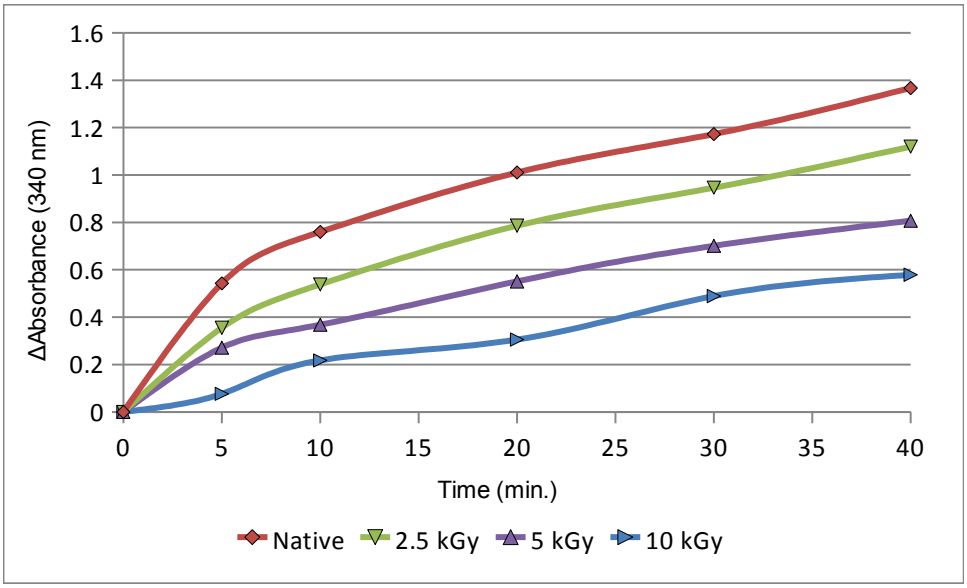

Figure 5: Absorbance readings at 340 nm for time-resolved digestions. Aliquots were removed at specific time points and diluted 10-fold in 4% $NaHCO_3$.

Figure 6 shows Lineweaver-Burk plots for the irradiation doses of pepsin. Lineweaver-Burk plots are a simplified representation of the kinetics of an enzyme under the Michaelis-Menten model. They are described by the following equation:

$$\frac{1}{v} = \frac{K_m[S]}{v_{max}} + \frac{1}{v_{max}}$$

This model assumes that the release of product from the enzyme occurs much faster than the formation of the enzyme-substrate complex, and that the total enzyme concentration does not change. The kinetic parameters K_m, v_{max}, and k_{cat} for pepsin at each dosage can be derived from the equations of the linear regressions. V_{max} is the maximum reaction velocity of the enzyme,

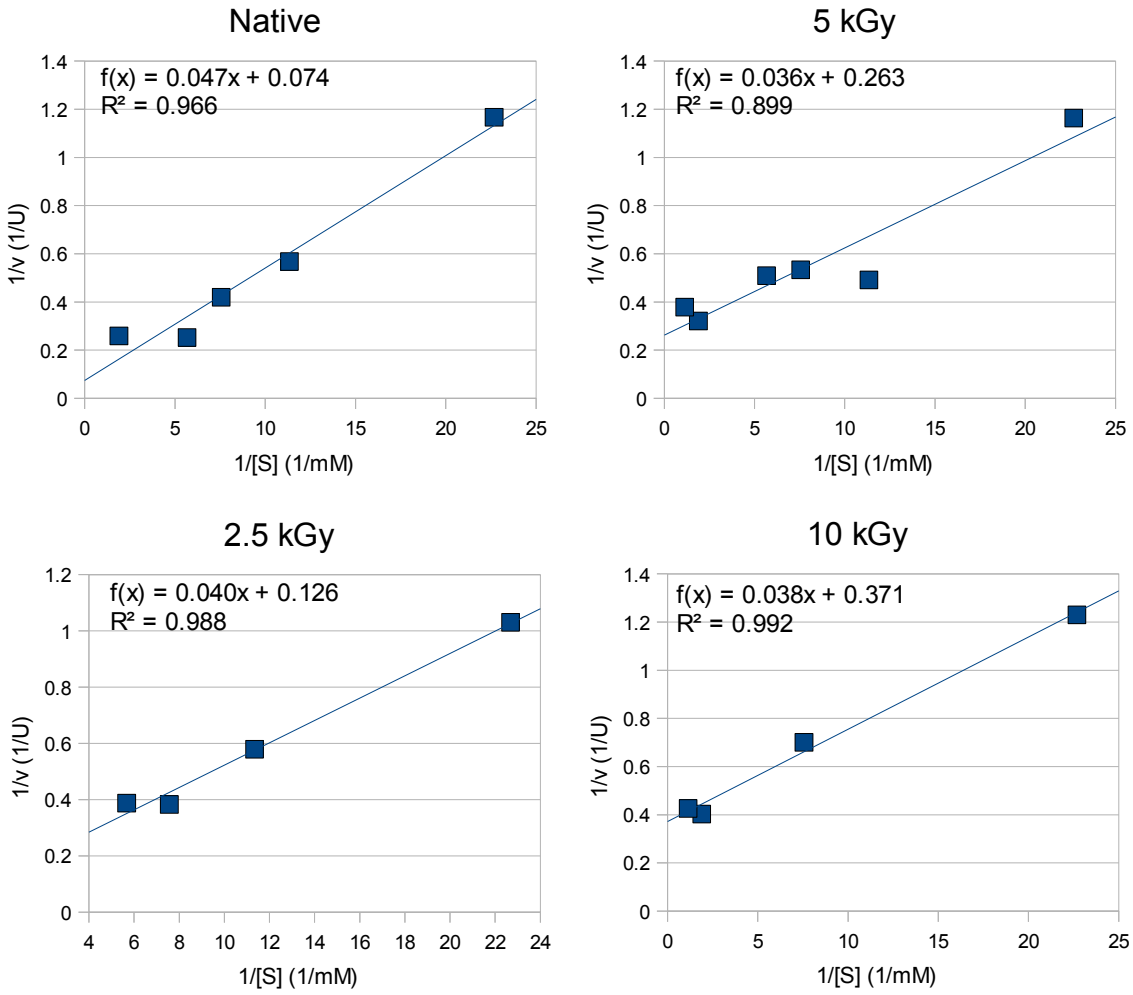

Figure 6: Lineweaver-Burk plots obtained from plotting the inverse of initial reaction velocity as a function of the inverse of substrate concentration.

obtained when the enzyme is fully saturated with substrate; this value can be derived from the y-intercept of the Lineweaver-Burk plot, which is equal to $1/v_{max}$. K_m is the Michaelis constant, and it is defined as the substrate concentration at which the enzyme has half of its maximum velocity, v_{max}; it can derived from the slope of the Lineweaver-Burk plot, which is equal to K_m/v_{max}, or the

x-intercept, which is equal to $-1/K_m$. k_{cat} is the turnover rate of the enzyme, given in the number of activity units per mole of enzyme; this value is found by dividing the v_{max} of an enzyme by the amount of enzyme in solution. These values are shown in Table 1. All parameters show decreases with increasing doses of radiation.

Dose (kGy)	K_m (mM)	V_{max} (U)	k_{cat} (U/mol)
0	0.81	13.5	9.35E+008
2.5	0.41	7.94	5.50E+008
5	0.18	3.8	2.63E+008
10	0.13	2.7	1.87E+008

Table 1: Different kinetic constants of enzyme solutions at each dose. Derived from Lineweaver-Burk Plots.

Figure 7 shows the results of performing SDS-PAGE on the irradiated pepsin solutions. While the 0 kGy lane has only a discrete band, a smear is apparent in the lanes for the irradiated samples. Irradiation has apparently created a solution with species that have a range of molecular weights.

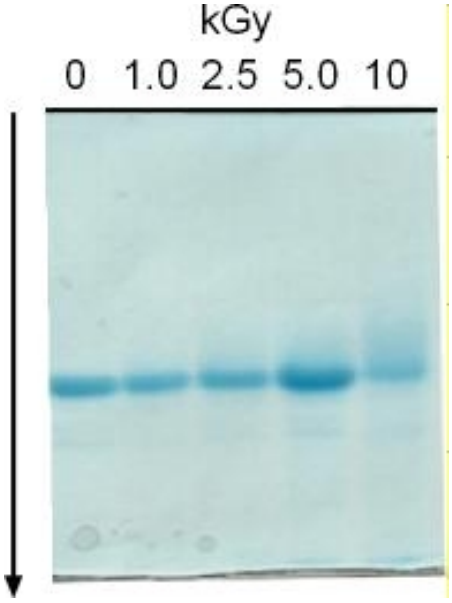

Figure 7: SDS-PAGE of irradiated pepsin solutions in a 10% gel.

Figure 8 is a graph of percent activity as a function of the dose; the k_{cat} value for pristine pepsin was considered 100% activity, and all the k_{cat} values of the other enzyme solutions were normalized to that value. A loss of activity

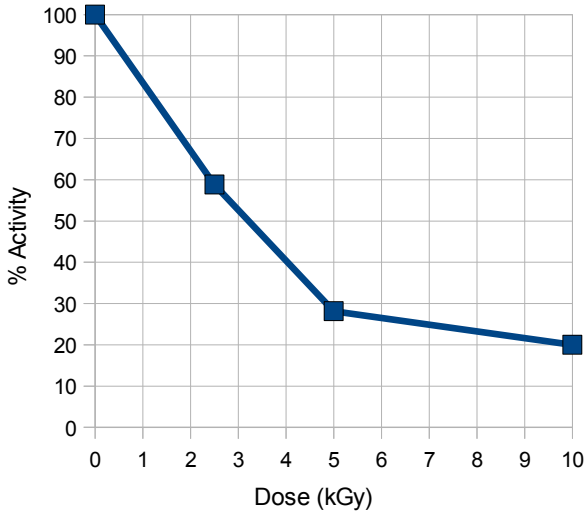

Figure 8: % Activity as a function of dose. k_{cat} for pristine enzyme was chosen as the 100% value.

Discussion

The goal of these experiments was to analyze the effects of radiation-induced radical reactions on the activity of pepsin. To that end, it was necessary to show that the enzyme was indeed active before irradiation ever occurred. Figure 2 unequivocally proves that the pepsin being used worked; the increase in absorbance at 280 nm indicated the presence of small, soluble peptides produced by the proteolytic action of pepsin on hemoglobin. As further proof of the enzyme's activity, absorption spectra were taken of the aliquots from the time-resolved digestions after they had been subjected to the spectrophotometric assay for primary amines (data not shown). At each time point, the absorption peak at 340 nm was taller than the last, showing that the pepsin was continuously digesting the ovalbumin. Figure 3 shows that free protein in solution specifically causes more intense absorption at 340 nm.

The standard curves shown in Figure 4 were necessary to equate changes in absorbance to changes in peptide content. Their linearity means that there is indeed a proportional relationship between the concentration of protein in solution and the intensity of the color in an assay tube. For more qualitative work, it's enough just to look at differences in absorbance. The changes in absorbance over time for the time-resolved digestions of ovalbumin (Figure 5) are the first sign that irradiation really did negatively affect the activity of pepsin. While each digestion began with comparable absorbances in the negative range, it's apparent that native pepsin displayed the best ability to cleave proteins. As the dose increased, the change in absorbance became less pronounced. Since an enzyme's function is directly tied to it's three-dimensional structure, this loss of activity indirectly supports the idea that irradiation really did have a significant effect on the structure and function of pepsin, either by scission or agglomeration.

Exploring the effects of irradiation on the kinetic parameters of pepsin gives a more quantitative picture of what happens. At a glance, the Lineweaver-Burk plots shown in Figure 6 indicate that the maximum velocity of the enzyme decreases as the dose increases. That is, the ability of the enzyme to initiate catalysis of a bound substrate was reduced. Table 1 shows this and two other important kinetic parameters, K_m and k_{cat}, both of which describe the kinetic behavior of an enzyme. K_m decreases as the dose increases, meaning that it takes fewer substrate molecules to completely saturate the enzyme. k_{cat} also decreases with irradiation, so the actual amount of substrate that can be cleaved in a given time is reduced.

The results from SDS-PAGE of the irradiated samples give further clues as to how pepsin could be inactivated by γ-radiation. Compared to the single, distinct band found in the lane for the native sample, all of the irradiated samples show a smear above the main bands; this indicates the presence of higher-molecular weight species that must be derived from the original, native pepsin. Unlike the previous experiments with ovalbumin that showed the presence of discrete aggregates[3, 6, 7], the smear is indistinct, suggesting that both scission and agglomeration were occurring at the same time. Such alterations in the 3D shape of pepsin could potentially affect access to the active site, the geometry of the active sight itself, or any domains important to the enzyme's function but not directly responsible for catalysis. Or if scission of the protein takes place prior to agglomeration, then there will already be a loss of activity.

Figure 8 shows a decrease in activity, the rate of which may decay over time since the concentration of pristine pepsin decreases throughout irradiation. The graph indicates a radiation dose of approximately 3, which corresponds to 1.62 mmol OH radicals produced per L of solution. Note that this is not concentration as the effective concentration depends on the dose rate; over the course of the irradiation, that 1.62 mmol will have been produced per mL. Dividing this by the concentration of pepsin during irradiation (0.289 mM) shows that it takes 18.7 OH radicals per molecule of pepsin to decrease enzyme activity by 50%.

In this experiment, the activity of pristine pepsin prior to irradiation was confirmed. It was demonstrated that irradiation of an aqueous solution of the enzyme led to a loss in its activity. Irradiation also led to the formation of higher-molecular weight agglomerates not present in a pristine solution. Since no other physical variables such as temperature or pH varied significantly, the loss in activity must be due to structural changes caused by the radiation chemistry.

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