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Influence of Macromolecular Crowding and Confinement on Enzyme Activity and Structure under Native and Denaturing Conditions

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Abstract

It is now well appreciated that both crowding and confinement influence enzyme structure and function due to excluded volume effects; however, the relative efficacies of these environments on protein fates remain unclear due to a lack of direct comparison studies. In this study, we explore the use of the biopolymer alginate to develop an *in vitro* platform to investigate the effects of both crowding and confinement on the behavior of two model enzymes - horseradish peroxidase and β -galactosidase. Alginate, in its solution phase, can be used as a crowding agent and, in its gel phase by crosslinking using divalent cations, to encapsulate and confine proteins, thereby allowing us to use the same system to directly compare the effects of crowding and confinement. Different degrees of crowding and confinement were achieved by varying the alginate concentration, and these studies demonstrated a clear dependence of enzyme activity on the degree of crowding and confinement. Moreover, our data also suggested that protein confinement in crosslinked alginate gels led to higher enhancements in enzyme activity under denaturing conditions relative to non-crosslinked crowded environments. Results from the kinetic analyses were corroborated using structural measurements of protein denaturation using the 8-anilino-naphthalene-1-sulfonic acid fluorescence assay.

Keywords: Protein crowding; Protein confinement; Enzyme activity; Enzyme structure; Protein denaturing conditions; Alginate solution and gel

Abbreviations

HRP: Horseradish Peroxidase; β -gal: β -Galactosidase; ANS: 8-Anilino-naphthalene-1-Sulfonic Acid; ABTS: 2,2'-Azino-Bis(3-Ethylbenzothiazoline-6-Sulphonic Acid); ONPG: o-Nitrophenyl- β -Galactoside; GdnHCl: Guanidine Hydrochloride

Introduction

In vitro investigations of protein structure and function have been most commonly based on simple buffer systems containing low concentrations of protein that consequently do not sufficiently replicate the highly crowded or confined intracellular environment of proteins [1-4]. Recently, several studies have therefore investigated protein behavior using *in vitro* conditions that more closely simulate the *in vivo* cellular environments, and supported that both confinement and crowding can significantly impact protein structure and function [5-12]. While crowding and confinement are often used synonymously, there is a significant difference in how the two environments exert their influence on proteins. Crowding refers to the presence of a high concentration of macromolecules that reduces the volume of solvent available to the proteins, while confinement arises from encapsulation of proteins in spaces only moderately larger than the proteins themselves [3,4]. Currently, theoretical analyses suggest that the two environments differ in their extent to which they affect protein behavior [3,4,13]. Experimental studies support the theory; however, the evidence has not been compelling, partly due to limited direct comparisons between the influences of these environments on

protein fates, particularly using the same system and similar measurement methods of protein structure and function [11,14]. It is therefore of fundamental interest to develop experimental conditions to investigate and compare the effects of crowding and confinement on protein structure and function.

In this study, we report the use of the biopolymer alginate in order to establish conditions for experimentally distinguishing between the effects of crowding and confinement, regarding their influence on protein structure and function. Alginate was used since it is a high molecular weight, anionic polysaccharide that can act as a crowding agent in its solution phase; moreover alginate can also be crosslinked using divalent cations to encapsulate and confine proteins, thereby allowing us to use the same system to study the effects of both crowding and confinement [15-17]. Furthermore, as with other crowding agents, alginate concentration can be varied to control the degree of crowding, as well as to control the pore size of the gel and thereby the degree of confinement. The choice of alginate as a confining agent is relevant, as previous research has shown that although alginate gels exhibit large heterogeneity in pore size, most of the pores range around 5-10 nm (especially for those prepared using prepared using concentrations $\geq 1\%$ w/v) [17-19]. In this study, we used three concentrations of alginate (1%, 5%, and 10% w/v) to study the roles of crowding and confinement on protein structure and function. The influence on protein function under both native and denaturing conditions was captured using kinetic assays on the model proteins, horseradish peroxidase (HRP) and β -galactosidase (β -gal) – two enzymes that have been routinely used to study the roles of various environments on protein function. These studies demonstrated a strong dependence of enzyme activity under native and denaturing conditions on the degree of crowding and confinement. Similar results were also observed for measurements of enzyme structure under

denaturing environments using the 8-anilino-naphthalene-1-sulfonic acid (ANS) fluorescence assay (as further detailed in the Results section). Furthermore, enzymes encapsulated in alginate gels were significantly more stable relative to enzymes in non-crosslinked solution-phase alginate, when exposed to denaturing environments. Our data agrees well with previous theoretical investigations of the effects of crowding and confinement on protein structure and function, which suggest that confinement leads to greater enhancements in protein activity under denaturing conditions relative to crowding [3,4,13]. To the best of our knowledge, this is the first experimental investigation demonstrating a clear dependence of enzyme activity and structure on crowding and confinement using the same system.

Materials and Methods

Materials

Horseshoe peroxidase (HRP) and β -galactosidase (β -gal), as well as all reagents for the quantification of enzyme structure and function were obtained from Sigma Aldrich (St. Louis, MO). Alginic acid (sodium salt, low viscosity, 4-12 cP, 1% w/v in H₂O at 25 °C) and calcium chloride for the crowding and confinement experiments were also purchased from Sigma Aldrich and used as received.

Experimental setup for confinement and crowding studies

For the confinement studies, enzymes were first encapsulated in alginate before conducting the spectroscopic measurements. Briefly, enzyme and alginate solutions in 100 mM pH 8.0 Tris-HCl buffer were combined to yield final alginate concentrations of 1%, 5%, or 10% w/v. 50 μ L of the enzyme-alginate mixture was pipetted into wells of a 96-well plate, followed by the addition of 100 μ L of 100 mM CaCl₂ in Tris-HCl buffer to crosslink the alginate. After 10 min, CaCl₂ solution was replaced with 100 μ L of Tris-HCl buffer containing no CaCl₂. For the crowding experiments, alginate stocks in 100 mM pH 8.0 Tris-HCl buffer were added to the enzyme assay mixtures such that the final alginate concentration was 1%, 5%, or 10% w/v alginate.

Enzyme kinetic assays

The initial reaction rates of alginate gel-encapsulated enzymes and enzymes in various concentrations of alginate were determined using a Tecan Infinite 200 PRO spectrophotometer (Durham, NC). HRP activity was determined by monitoring H₂O₂ mediated oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) at 420 nm; HRP concentration was 90 nM, and ABTS and H₂O₂ concentrations were 10 μ M and 40 μ M, respectively. To estimate the Michaelis-Menten kinetic constants, the concentration of ABTS was varied between 0-30 μ M, while keeping the concentrations of HRP and H₂O₂ constant. The reaction rates were then fitted to a non-linear regression model using GraphPad Prism to determine K_M and V_{max}. β -gal activity was measured by monitoring the hydrolysis of o-Nitrophenyl- β -galactoside (ONPG) to o-nitrophenol at 420 nm; β -gal concentration was 10 nM and the ONPG concentration was 80 μ M. Initial rates of ABTS oxidation and ONPG hydrolysis by alginate gels and solutions containing no enzyme were measured as controls. To determine enzyme activity in the presence of the denaturant - ethanol, enzymes in various alginate solution- and gel-phase conditions were first equilibrated in buffer solutions containing 35% ethanol for ca. 5 minutes, followed by measurement of initial reaction rates (35%

ethanol was used based on our preliminary experiments that indicated that this concentration strongly inhibited enzyme function). We confirmed, by comparing the viscosity of alginate solutions prepared using standard buffer and 35% ethanol, that the addition of ethanol did not affect alginate solution properties. We also performed rheological characterization of alginate gels soaked in buffer and 35% ethanol for ca. 30 min and did not notice any significant differences between the elastic properties of buffer- and ethanol-soaked samples (data not shown). For thermal denaturation experiments, the various enzyme formulations were maintained at 80 °C for different periods of time and then cooled to room temperature in an ice bath before measuring the initial rates at room temperature. Enzyme assays were also performed after incubating stock solutions of HRP and β -gal with 100 mM CaCl₂ for 10 min to confirm that the addition of CaCl₂ (for enzymes in alginate gel preparations) did not impact enzyme reaction rates. We also confirmed, by exposing HRP- and β -gal-containing alginate gels to buffer solutions for different periods of time (up to 60 min) that the enzymatic activities of the leachate was <10% of those of the alginate gel-encapsulated enzymes (data not shown).

ANS fluorescence assays

ANS fluorescence emission spectra (between 450 and 550 nm) of enzymes in various alginate solution- and gel-phase conditions in standard Tris-HCl buffer conditions and denaturing conditions were collected after excitation at 360 nm using the Tecan Infinite 200 PRO spectrophotometer. The enzyme formulations were first equilibrated in either Tris-HCl buffer or chemical denaturant conditions (i.e. in buffer solutions containing either 35% ethanol or 6 M GdnHCl) for ca. 5 minutes prior to the fluorescence measurements. For thermal denaturation experiments, the various enzyme formulations were maintained at 80 °C for 60 minutes and then cooled to room temperature in an ice bath before the fluorescence measurements. The final protein concentration was 40 μ g/mL for all the measurements and the ANS concentration was 13.5 μ M. ANS fluorescence spectra of alginate solutions and gels containing no enzyme were recorded similarly and subtracted from the spectra of enzyme (please note that both solution- and gel-phase alginate had minimal interference with the fluorescence measurements).

Results

Kinetic parameters of enzymes in crowded and confined environments

Alginate, a naturally occurring high molecular weight polysaccharide that can form hydrated gels in the presence of multivalent cations [17], was chosen as a flexible platform to explore the roles of crowding and confinement on protein structure and function. We first investigated the role of such environments on enzyme kinetic parameters. Figure 1 shows the initial reaction rates of HRP and β -gal in alginate solution- and gel-phase conditions for various concentrations of alginate relative to enzymes in standard Tris-HCl buffer. The data indicated a decrease in initial rates of the enzymes for alginate solution- and gel-phase conditions in an alginate concentration dependent manner. Excluded volume due to crowding or confinement arguments alone cannot be used to explain these results, since excluded volume effects have been shown to increase effective enzyme concentration and thereby enzymatic reaction rates [2,3].

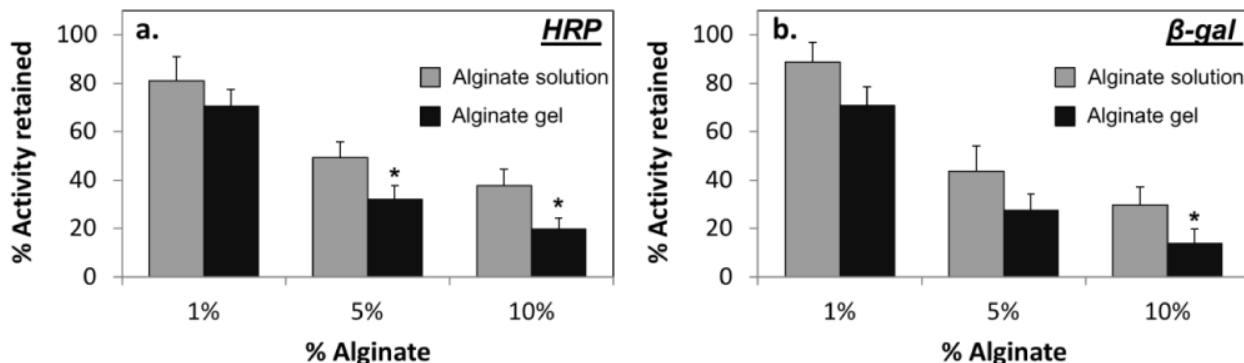


Figure 1: Initial rates of (a) HRP and (b) β -gal in alginate solutions (grey bars) and alginate gels (black bars) for various concentrations of alginate relative to the respective enzymes in buffer. Data shown are the mean of triplicate measurements plus standard deviation. * $P < 0.05$ relative to the values obtained for alginate solutions as determined by Student's unpaired two-tailed t test. The experiments were repeated at least three times with similar results.

However, our data is not entirely surprising as previous studies have attributed decreases in initial rates for enzymes under similar conditions to diffusion-controlled enzyme kinetics [4,9,12,20-22].

We performed additional kinetic analysis to estimate the apparent Michaelis-Menten kinetic constants K_M and V_{max} for HRP in alginate solution- and gel-phase conditions (Table 1). These studies revealed an alginate concentration dependent increase in the K_M values for HRP in alginate solutions and gels compared to those for HRP in standard buffer conditions. The observed increases in the K_M values in the alginate based-crowded and confined environments can be attributed to previously established roles of decreased enzyme and substrate diffusion [9,22]. We also observed slight decreases in the values of

V_{max} for HRP in alginate conditions, which is inconsistent with previous literature suggesting that an increase in the effective enzyme concentration due to excluded volume effects can lead to increases in V_{max} [4,22]. However, our results are consistent with literature that explains a decrease in V_{max} due to either conformational changes of the enzyme active site or increase in effective inhibition by the product [9,23,24]. More importantly, it is noteworthy that the differences in apparent K_M as well as the initial rates and V_{max} (relative to enzymes in buffer) were more significant for gel-encapsulated enzymes than for enzymes in alginate solutions, providing additional evidence that alginate gels and alginate solutions present different environments to the proteins.

Condition	Solution phase		Gel phase	
	V_{max} (nM s ⁻¹)	K_M (μ M)	V_{max} (nM s ⁻¹)	K_M (μ M)
0% alginate	10.3 \pm 0.3	4.8 \pm 0.6	n/a	n/a
1% alginate	9.9 \pm 0.4	5.1 \pm 0.4	9.3 \pm 0.3	5.8 \pm 0.5
5% alginate	8.4 \pm 0.9	7.9 \pm 1.0	7.6 \pm 0.4	8.4 \pm 0.7
10% alginate	7.9 \pm 0.4	8.8 \pm 0.4	6.4 \pm 0.5	10.1 \pm 0.6

Table 1: Kinetic constants of ABTS oxidation by HRP in alginate solutions and gels.

Effect of degree of crowding and confinement on enzyme activity under denaturing conditions

Next, we proceeded to evaluate the roles of crowding and confinement on enzyme activity under denaturing conditions. For this, we compared the initial rates of the various HRP and β -gal formulations in standard buffer and buffer containing 35% (w/v) of the denaturant ethanol. Ethanol was chosen as the protein denaturant [25], instead of the more widely used guanidine hydrochloride (GdnHCl) [26,27], because GdnHCl interfered with the experiments used to measure enzyme activity (i.e. the ABTS and ONPG enzyme assays). Figure 2 clearly shows that both crowding and confinement led to

higher retention of enzyme activity relative to the no alginate enzyme control (i.e. enzymes in buffer) and that the degree of stabilization was strongly dependent on alginate concentration. Additionally, these results also revealed enhanced stabilization for alginate gel-encapsulated enzymes relative to enzymes in alginate solutions for all concentrations of alginate tested. These trends, i.e. higher enzyme activity in confined vs. crowded environments were also observed for thermal denaturation (Figure 3). Thus, our experimental analyses of enzyme activity under denaturing conditions strongly suggest that confinement leads to higher enhancements in protein stability relative to crowding.

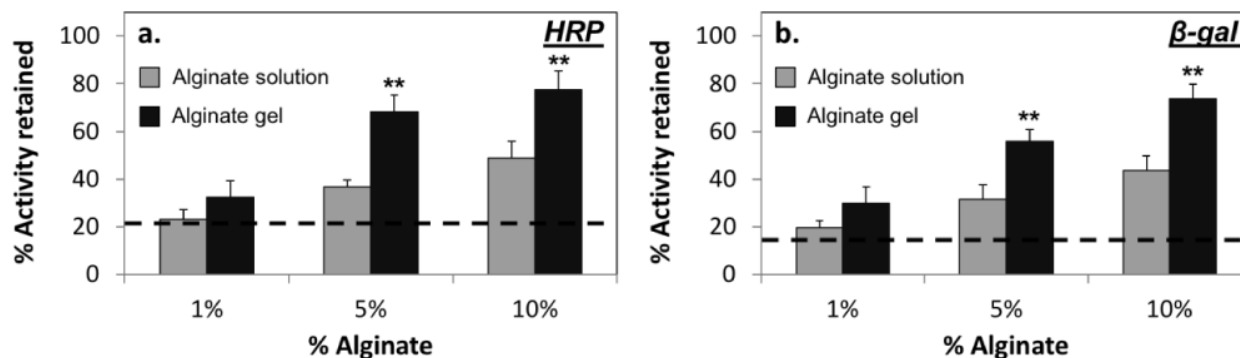


Figure 2: Percent activity retained for (a) HRP and (b) β -gal in alginate solutions (grey bars) and alginate gels (black bars) for various concentrations of alginate in the presence of 35% ethanol. The relative activities were calculated by normalizing the activity in the presence of 35% ethanol to the activity in buffer containing 0% ethanol. Data shown are the mean of triplicate measurements plus standard deviation. * $P < 0.05$ and ** $P < 0.01$ relative to the values obtained for alginate solutions as determined by Student's unpaired two-tailed t test. The experiments were repeated at least three times with similar results. The horizontal dashed line represents the average percent activity retained for the enzymes in buffer exposed to the chemical denaturation conditions.

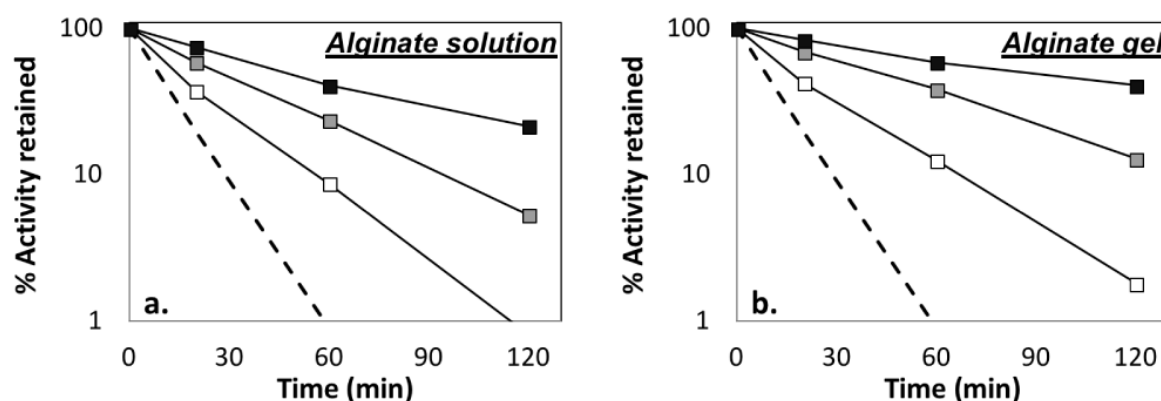


Figure 3: Time-dependent deactivation of HRP in buffer (dashed line) and HRP in (a) alginate solutions and (b) alginate gels exposed to 80°C for various concentrations of alginate – 1% (white squares, solid line), 5% (grey squares, solid line), and 10% (black squares, solid line). Values for activity retained were calculated by normalizing activities at various time points to the initial activity at $t = 0$ min. Data shown are the mean of triplicate measurements (with standard deviation $< 10\%$). The experiments were repeated at least three times with similar results.

Enzyme structure under denaturing conditions in crowded and confined environments

Having established the roles of crowding and confinement on enzyme initial rates and the retention of enzyme activity under denaturing conditions, we wished to confirm that the functional stability was correlated with retention of protein structure. In order to compare the effects of protein crowding and confinement on enzyme structure under denaturing environments, we used 8-anilino-1-naphthalenesulfonic acid or ANS-binding fluorescence assay. ANS has been previously used as a sensitive fluorescent probe for the detection of partially folded or fully unfolded proteins; fluorescence of ANS increases substantially upon binding to hydrophobic regions of proteins that become exposed during denaturation [28-31].

Consistent with the kinetic studies, when we exposed the various alginate-enzyme formulations to denaturing conditions, we observed

that the increases in the ANS signal were strongly dependent on alginate concentration. Specifically, when exposed to 35% ethanol, significant increases in ANS fluorescence were observed for enzymes in standard buffer conditions and low concentrations of alginate (for both crowding and confinement), while only a modest increase in the ANS signal was observed for enzymes in 10% alginate (Figures 4 and 5). Furthermore, consistent with the aforementioned investigations of enzyme activity, the ANS studies indicated that confinement lead to higher enhancements in enzyme structural stability relative to crowding. Additional experiments that tested denaturation induced by GdnHCl and high temperature under various conditions also showed similar trends, i.e. enhanced retention of enzyme structure in alginate based-confined conditions relative to crowded conditions (Figure 5).

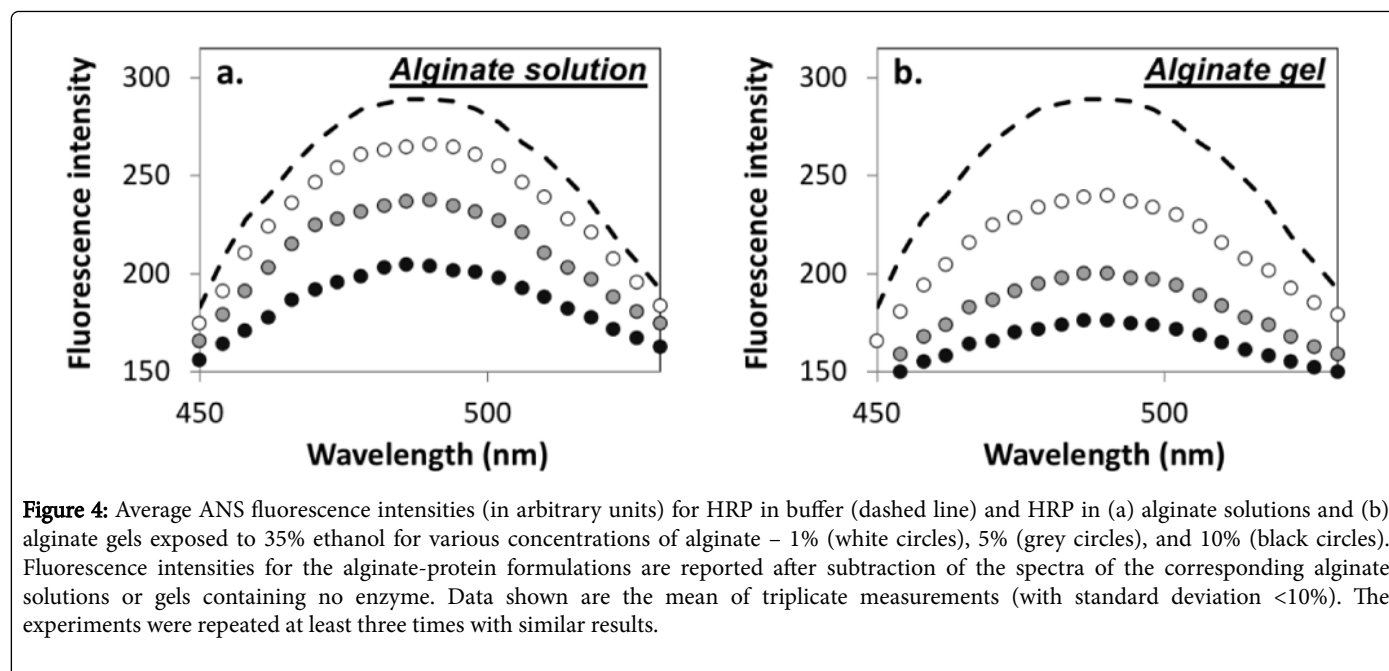


Figure 4: Average ANS fluorescence intensities (in arbitrary units) for HRP in buffer (dashed line) and HRP in (a) alginate solutions and (b) alginate gels exposed to 35% ethanol for various concentrations of alginate – 1% (white circles), 5% (grey circles), and 10% (black circles). Fluorescence intensities for the alginate-protein formulations are reported after subtraction of the spectra of the corresponding alginate solutions or gels containing no enzyme. Data shown are the mean of triplicate measurements (with standard deviation <10%). The experiments were repeated at least three times with similar results.

Discussion

Majority previous studies exploring the mechanisms behind protein structure and function have been performed in simple buffer systems with low concentrations of the test protein. However, *in vivo* proteins have evolved to function in more complex, highly crowded or confined environments [1-3]. Therefore, recent research has attempted to mimic the complexity of *in vivo* environments in order to improve our understanding of protein behaviors. Crowding and confinement are related in that both the environments influence protein conformation and dynamics by reducing the volume accessible to the protein molecules [2,3,11,32]. However, they are distinct in that crowding refers to effects of volume exclusion arising from the presence of other soluble macromolecules, while confinement refers to effects due to the presence of a fixed, impenetrable boundary. This distinction can lead to important differences in how crowding and confinement affect protein fates [3,4,11,32]. For example, using theoretical analyses, it has been estimated that confinement can lead to higher enhancements in protein stabilization relative to crowding [3,4,13]. However, these theoretical analyses are not well supported by experimental evidence. Specifically, while several research groups have presented evidence that both these environments can positively influence protein stability and dynamics [4,11,13,14], direct experimental comparisons between the influences of crowding and confinement on protein structure and function have been limited [11,14].

In this study, we investigated the effects of crowding and confinement on enzyme structure and function in physiological buffer and denaturing conditions using alginate formulations that allowed us to experimentally distinguish between the effects of crowding and confinement on protein behavior. Alginate is a high molecular weight polysaccharide and therefore can be used as a macromolecular crowding agent. Alginate can also be physically crosslinked using divalent cations to form a gel and encapsulate proteins to mimic the effects of confinement. Our data revealed higher enhancements in protein activity under denaturing environments due to confinement relative to crowding, which is in good agreement with previous theoretical investigations of the effects of crowding and confinement

on protein structure and function [3,4,13]. Moreover, our studies also indicated that the degree of crowding and confinement, varied by changing the concentration of alginate, also strongly influences protein activity under denaturing environments. It may be argued that the observed differences in the activities of the proteins in alginate solutions and gels are due to the differences in excluded volume effects arising from variances in the net negative charge of alginate in solution and calcium-crosslinked gel phases. However, it is important to note that we see similar effects of crowding and confinement for the two proteins with different isoelectric points (pI of HRP=8.8 and pI of β -gal=4.2) used in the study, thereby indicating that the effect of changes in net charge of alginate on the observed results, if any, are minimal. Finally, the enzyme activity measurements were supported using ANS measurements of protein denaturation that revealed higher enhancements in structural stability for enzymes in confined conditions relative to crowding. Although ANS fluorescence only provides an indirect readout of protein structure, we used the assay to measure relative changes in protein structure (denaturing conditions relative to buffer conditions), and therefore have confidence in the conclusions drawn from the data. Future efforts will focus on stability measurements using more direct approaches, including fluorescence and circular dichroism spectroscopy.

The outcomes presented in this paper should be of interest to several areas of research, and in particular, to theoreticians and experimentalists investigating the role of *in vivo* and *in vivo*-like environments on protein fates and behavior. In what we believe is the first study of its kind, we directly examined the effects of crowding and confinement on protein structure and function under native and denaturing environments. The observed differences can be explained by previous theoretical studies that indicate that inherent differences in crowding and confinement, i.e. volume exclusion vs. fixed boundary effects, lead to differences in protein fates [3,4,13,32]. These studies have also proposed that, compared to crowding, protein confinement may lead to decreased folding rates for the proteins. This supports our observations that demonstrate greater retention in protein activity under denaturing conditions for confinement relative to crowding.

Furthermore, several studies have indicated protein unfolding and aggregation as major causes of common diseases including cataract, diabetes, and various neurodegenerative disorders [33-35]. Since crowding and confinement can significantly impact these protein fates, developing *in vitro* platforms to evaluate the role of such environments on protein behavior is also of relevance to human health and medicine [36-39].

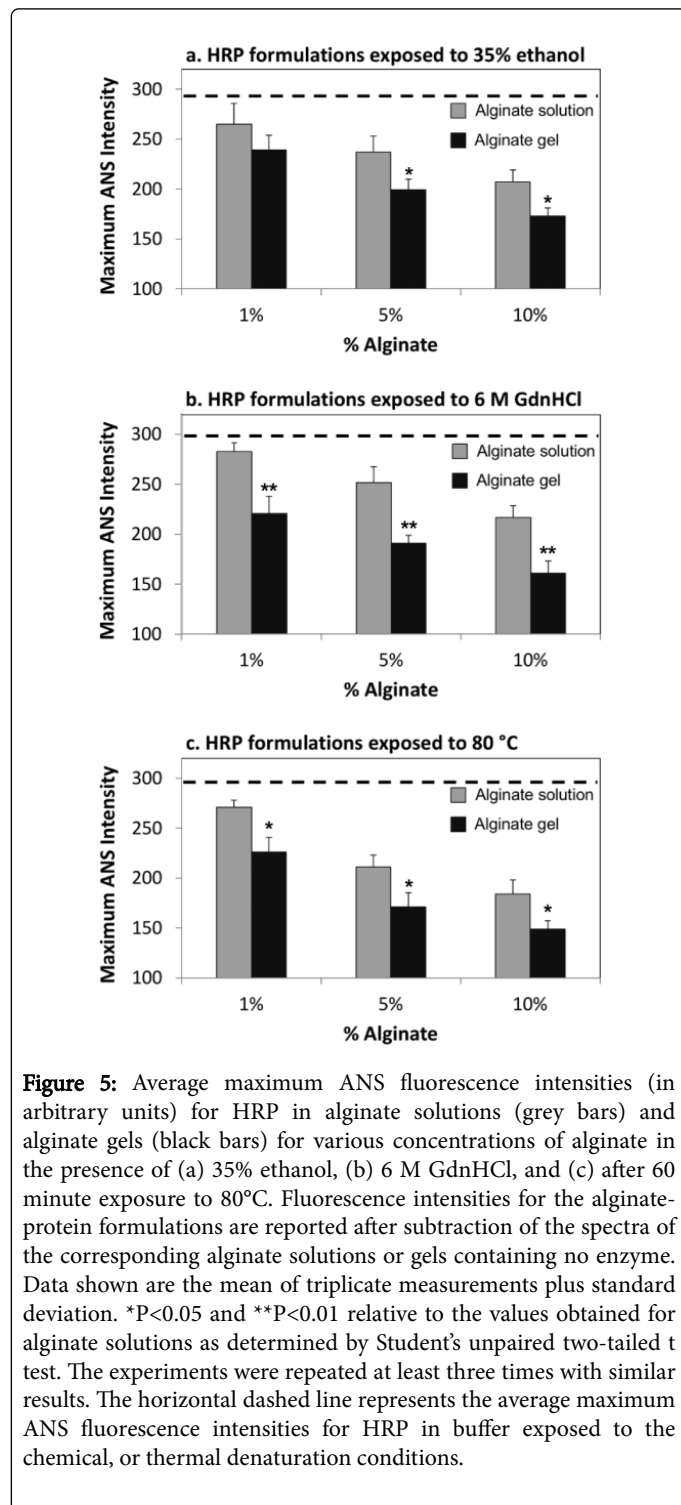


Figure 5: Average maximum ANS fluorescence intensities (in arbitrary units) for HRP in alginate solutions (grey bars) and alginate gels (black bars) for various concentrations of alginate in the presence of (a) 35% ethanol, (b) 6 M GdnHCl, and (c) after 60 minute exposure to 80°C. Fluorescence intensities for the alginate-protein formulations are reported after subtraction of the spectra of the corresponding alginate solutions or gels containing no enzyme. Data shown are the mean of triplicate measurements plus standard deviation. *P<0.05 and **P<0.01 relative to the values obtained for alginate solutions as determined by Student's unpaired two-tailed t test. The experiments were repeated at least three times with similar results. The horizontal dashed line represents the average maximum ANS fluorescence intensities for HRP in buffer exposed to the chemical, or thermal denaturation conditions.

Conclusions

In conclusion, in this paper we exploited the ability of alginate, a high molecular weight polysaccharide, to form ionically crosslinked gels to study the effects of both crowding and confinement on enzyme activity. Moreover, by altering the alginate concentration, we also varied the degree of crowding and confinement. These studies strongly indicated enhanced enzyme activity under denaturing conditions in confined environments relative to crowded environments, as well as due to increases in the degrees of both crowding and confinement. Taken together, the data presented in this paper significantly contributes to the growing literature of theoretical and experimental studies attempting to understand the role of *in vivo* cellular environments on protein behavior.

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