Rheo-NMR Studies of an Enzymatic Reaction: Evidence of a Shear-Stable Macromolecular System

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ABSTRACT Understanding the effects of shear forces on biopolymers is key to understanding how biological systems function. Although currently there is good agreement between theoretical predictions and experimental measurements of the behavior of DNA and large multimeric proteins under shear flow, applying the same arguments to globular proteins leads to the prediction that they should only exhibit shear-induced conformational changes at extremely large shear rates. Nevertheless, contradictory experimental evidence continues to appear, and the effect of shear on these biopolymers remains contentious. Here, a custom-built rheo-NMR cell was used to investigate whether shear flow modifies enzyme action compared with that observed quiescently. Specifically, ^{1}H NMR was used to follow the kinetics of the liberation of methanol from the methylesterified polysaccharide pectin by pectinmethylesterase enzymes. Two different demethylesterifying enzymes, known to have different action patterns, were used. In all experiments performed, Couette flows with shear rates of up to 1570 s^{-1} did not generate detectable differences in the rate of methanol liberation compared to unsheared samples. This study provides evidence for a shear-stable macromolecular system consisting of a largely β-sheet protein and a polysaccharide, in line with current theoretical predictions, but in contrast to some other experimental work on other proteins.

INTRODUCTION

Biopolymers under shear

Investigating how biopolymers respond to applied forces is vital for understanding how cellular and extracellular structures function in the real world (1). Force signaling in biological systems is a topical and important area in modern molecular biophysics, and is used, among other things, to elucidate how shear modulates cellular function (2,3). The advent of single-molecule force spectroscopy has permitted the measurement of the stretching response of many biopolymers to directly applied forces in the piconewton–nanonewton range, and continues to yield fascinating insights into force-driven conformational changes. However, although it has been addressed experimentally for a much longer time, the closely related question of whether shear stresses imparted by flow fields might also significantly alter biopolymer conformation has not been unequivocally answered.

That is not to say that there are no experimental studies in this area that can claim to be reasonably well understood or supported by current theory. Indeed, a decade ago, the configurations of fluorescently labeled λ bacteriophage DNA (λ-DNA) were investigated under uniform shear flow with the use of single-molecule imaging techniques, and the results showed both elongation and tumbling of the polymer (4), in agreement with the predictions of polymer physics for chains in which solvation is preferred to self-association (4,5). More recently, experiments on the large multimeric protein assembly Von-Wildebrand factor (VWF), motivated by its role in the circulatory system, highlighted shear-directed unfolding at a critical shear rate as a mechanism not only for mediating platelet adhesion (6), but also for controlling the accessibility of the macromolecular complex to its attendant enzymes (7). In response to the observation of this critical unfolding, theoretical work using Brownian dynamics simulations (BDS) and scaling arguments was undertaken. By considering the effect of the competition between hydrodynamic drag and restoring interaction forces on thermally excited protrusions of polymeric globules, investigators were able to predict that indeed unfolding occurs at a critical shear rate (8), as found experimentally for VWF, and in sharp contrast to the good solvent model applicable to DNA. These models have since been extended to the consideration of surface-enhanced unfolding (9) and the effects of the solvent penetration depth (10). Of interest, when one includes hydrodynamic interactions in the model instead of assuming that the polymer chain drains freely, the manner in which the critical shear rate for unfolding depends on the globule size changes drastically. Predictions are successful in reproducing the experimental features of VWF unfolding, including the size of the monomeric units that are required for unfolding at physiologically relevant shear rates (8). In VWF these units are polymeric globules, rather than single amino acids, and thus the intact domains of the multimeric assembly are pulled away from each other in shear flow while their local structure remains largely unperturbed.

Applying the same theory that explains VWF behavior so well to monomeric collapsed proteins suggests, however, that critical shear rates on the order of 10^8 s^{-1} would be required for unfolding. Similar work employing BDS

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simulations in which a Go-like implementation (11) of the coarse-graining was applied to specifically model ubiquitin and integrin (12) yielded similar conclusions, i.e., short-lived metastable intermediates were predicted rather than smooth unfolding, but only at large shear rates that are typically unattainable in the laboratory. Some experimental studies have suggested that indeed there is no significant effect from shearing certain globular proteins (13–17) (up to 10^5 s^{-1} in one study (17), where it was also argued that something on the order of 10^8 s^{-1} would be required to induce conformational changes).

In contrast, a number of other studies have provided evidence that the conformation of certain proteins can be modified by the application of considerably lower shear rates (18–20), which at present would appear to contradict theoretical predications. It has been convincingly argued that the results of several earlier studies in particular were likely to reflect the presence of air/water interfaces, bubble entrainment, exposure to solid surfaces, contamination with particulates, or pump cavitation (16). In particular, it is worth noting that interfacial forces are much closer in magnitude to those found to be active in conformational manipulation in atomic force microscopy and optical tweezers experiments, compared with those that might be expected from shear flows (16). Nevertheless, recent well-controlled studies using modern optical and spectroscopic methods have also reported conformational changes in monomeric proteins (21–26). A resolution of this issue is needed and could have far-reaching implications, such as for understanding the genesis of amyloidic diseases (24).

It seems, then, that further experimental work in the area is required, and indeed that the introduction of new techniques that possess different sensitivities and provide complementary information is warranted. Here, we studied the effect of shear on an enzymatic reaction (specifically, the demethylesterification of the polysaccharide pectin with pectinmethylesterase (PME)) in situ using rheo-NMR. The shear rates accessible in our experiments (10^2–10^3 s^{-1}) are orders of magnitude below those expected to alter the conformation of our enzyme based on current theories as described above, and although polysaccharides under shear have received little specific consideration, it is likely that a good solvent (as opposed to globule) model would be applicable. In this case, gradual elongation of the substrate might be expected, but only at shear rates approaching the reciprocal of the relaxation time, which can be estimated as the longest Rouse mode (27) from the contour and persistence lengths as <1 μs. Therefore, even though current theories clearly do not predict that differences will be manifest using the shear rates achievable herein, it should be noted that this null prediction also holds for the above-mentioned systems that have yielded positive results. By adding further well-controlled data obtained with a different technique, we hope to move toward an understanding of whether the seemingly contradictory findings can eventually rationalized in terms of the differences in cell designs, detection modalities, or the specific proteins studied.

For the selected system, successful visits by the enzyme to the polysaccharide chain leave a marker on the processed sugar rings in the form of a deesterified residue. This process can readily be followed in situ by using NMR spectroscopy to monitor the concentration of the reaction product (methanol) with time. Although it is possible that the shear stability of the enzyme-substrate complex is different from that of the component macromolecules, we reasoned that shear-induced conformational changes to either the enzyme or the substrate would affect the reaction kinetics by modifying the transport or binding behavior. Furthermore, if changes are observed in enzyme rates under shear, then changes in the way in which the macromolecular interaction was modified will be retained in the resulting sequence of deesterified residues, and this sequence can be measured by a number of experimental approaches. In principle, NMR could be used to detect shear-induced conformational changes directly via changes in enzyme or substrate chemical shifts. However, this was not feasible in real time because of 1), the congestion of the 1D ^{1}H spectra (these spectra are essentially of the polysaccharide substrate only, owing to the low concentration of the enzyme used, and are not highly resolved at room temperature); and 2), the time required to obtain a 2D spectrum with an adequate signal/noise ratio. However, if changes in the enzyme kinetics are detected, further experiments can be designed and optimized to exploit the molecular detail afforded by NMR spectroscopy. We investigated two enzymes, as described below.

Specific enzyme system under study

The substrate: pectin

Pectin is a complex heterogeneous polysaccharide that plays an important role in controlling the mechanical strength and flexibility of the primary cell wall of land plants. The dominant structure of pectin is a linear chain of poly-α-(1-4)-linked *D*-galacturonic acid, which forms homogalacturonan. Pectin's functionality varies significantly depending on its fine structure, which is largely controlled by the pattern and degree of methylesterification (DM) of the carboxyl groups on the homogalacturonan (28,29). Commercially, pectin is widely used in both food and pharmaceutical industries as a gelling agent. Commercial pectins contain large amounts of homogalacturonan components that can be easily methyldeesterified or manipulated to form pectins with desired properties; however, pectins found in plant cell walls are a complex mixture of homogalacturonan and other polysaccharides, such as rhamnogalacuturonan I and II, that are designed to meet specific needs within the plant.

The enzymes: PMEs

PMEs are enzymes that are found in all plants, as well as in bacteria and fungi. Their main role in pathogens is to digest

cell walls, whereas in plants they remodel the mechanical properties and porosity of the cell wall by evoking changes in the pectin structure and thus in the pectin's calcium-mediated cross-linking ability. Specifically, PME catalyzes the deesterification of methylesterified *D*-galacturonic acid components, liberating methanol. PMEs from plants, bacteria, and fungi form part of carbohydrate esterase family 8. They are typically ~30 kDa in size and are referred to as parallel β-helix proteins, with the β-prism organization consisting of three parallel β-sheets (30–32).

PMEs are generally reported to follow a Michaelis-Menten model, with a multitude of isozymes having different pH optima and kinetic properties (28). The Michaelis constant, K_m, varies widely depending on the type of substrate and on environmental conditions such as pH, temperature, and ionic strength, but generally lies between 10^{-1} and 10^{-7} M (33). Plant PMEs show optimal performance in the pH range of 6.5–9.5, whereas fungal PMEs show optimal performance under acidic conditions (pH 4.0–5.2) (28). It has been reported that PME does not act on a methylesterified component that is located between two deesterified carboxyl groups, and does not completely deesterify pectin, stopping at a certain degree of esterification governed presumably by the requirement of a particular binding epitope (34–38).

Plant PMEs

It has been reported that plant PMEs (pPMEs) initiate action on a methylesterified carboxyl group adjacent to an already deesterified carboxyl group, attracted by its charge, and then progressively remove the neighboring methylester components on the pectin chain (28,30). This can result in areas containing long stretches of deesterified galacturonic acid components on pectin chains. pPME action is conventionally described by a multiple attack action mechanism whereby the enzyme catalyzes the transformation of a limited average number of residues at a time on one pectin chain (39); however, this is likely to depend on the nature of the substrate, and the precise mechanism of action remains to be determined (30,40).

Fungal PMEs

In contrast to pPMEs, fungal PMEs (fPMEs) do not work processively; rather, they act via a multiple-chain mechanism. This means that the enzymes dissociate after each reaction, resulting in a single-residue attack (39) and a random removal pattern of methylester groups (41). The pPME and fPME enzymes then move along the substrate differently and likely bind the polysaccharide chain with different strengths, all of which could conceivably alter sensitivity to shear.

MATERIALS AND METHODS

RheoNMR cell

Shear flow measurements were carried out using a custom-built NMR shear cell with Couette geometry, which consists of two concentric NMR tubes of different diameters with the sample contained in the annulus (Fig. 1). The inner tube can be connected via a rigid shaft to a programmable stepper motor, which rotates to create a velocity gradient across the sample. The maximum and average shear rates applied to the sample were calculated using the following equations:

$$\dot{\gamma}_{\max} = \frac{2\omega}{1 - \left(\frac{R_i}{R_o}\right)^2} \tag{1}$$

and

$$\dot{\gamma}_{av} = \frac{4\omega\left(\frac{R_i}{R_o}\right)^2 \ln\left(\frac{R_i}{R_o}\right)}{\left(1 - \left(\frac{R_i}{R_o}\right)^2\right)^2} \tag{2}$$

where ω is the angular velocity of the rotating inner tube, and R_i and R_o are the inner and outer radius of the gap, respectively (42). For control experiments (i.e., without shear) an identical cell was used, but without the stepper motor hardware. Beyond a critical shear rate, vibration from the drive shaft was found to affect the NMR spectra. Therefore, for experiments at the

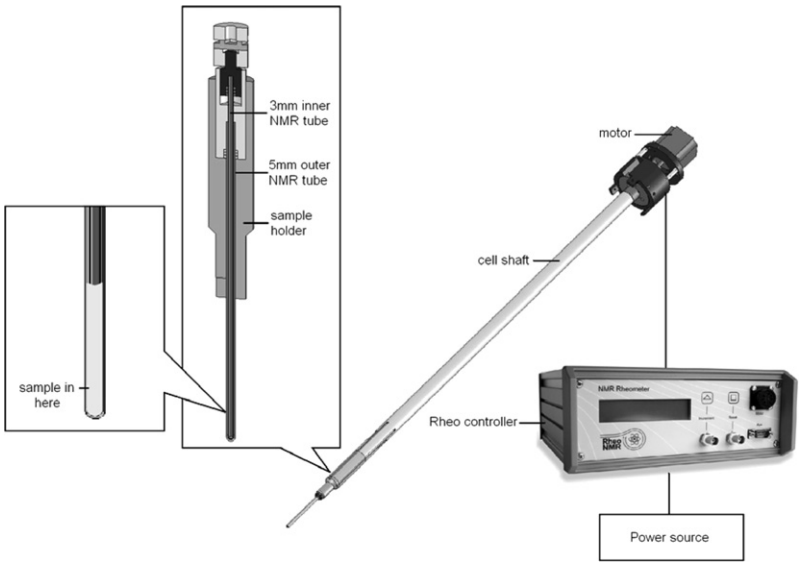

FIGURE 1 Schematic of the NMR couette shear cell used in this work, illustrating (*left to right*) the sample tube, its incorporation into a spinner-type housing with the inner tube attached to an external coupling, and the attachment of this housing to the cell shaft, which could be introduced in the magnet bore and driven to rotate via the stepper motor.

highest shear rates, a benchtop cell of essentially identical design was used. The drive shaft of this apparatus was considerably shorter because it did not have to be inserted into the spectrometer, which allowed significantly higher shear rates to be obtained. Samples from this cell were subsequently transferred to a conventional NMR tube for analysis. In summary, accessible average shear rates for in situ experiments in the NMR spectrometer were up to 240 s^{-1} (with the corresponding maximum experienced being 350 s^{-1}), whereas the benchtop cell allowed ~7-fold greater shear rates to be achieved. It should be noted that not only do these shear rates span the range used in previous studies that reported positive changes in macromolecular conformation, they are also physiologically relevant. In the circulatory system, shear rates can vary over orders of magnitude of $1–10^5$ (43) (with $10^2–10^3$ s^{-1} more commonly quoted). The best available estimates of shear rates experienced in phloem transport in vascular plants are also in this range, based on the application of Poiseuille's equation and the size of sieve pores (D. Renard, Institut National de la Recherche, personal communication, 2009). For the highest shear rate applied in the in situ experiments, the Reynolds number for the cell is ~80, and the formation of Taylor vortices is not expected (although this is a possibility in the benchtop cell (45)).

Pectin

Pectin extracted from apples was purchased from Fluka Biochemika (Buchs, Switzerland), with a DM value of 70–75%. The molecular mass was ~30–100 kDa.

Enzymes

pPME (Sigma-Aldrich, Auckland, New Zealand) was prepared as a 0.05% w/v stock solution using MilliQ water. fPME (a pure isozyme from *Aspergillus niger*) was kindly supplied by Jacques Benen (46) as a $3.75 \times 10^{-3\%}$ w/v solution in acetate buffer.

Sample preparation

Pectin was dissolved at 323 K in 1 mL of an appropriate buffer (100 mM sodium phosphate/deuterium oxide (D_2O, 99.9%; Merck) at pD 7.4 for experiments with pPME, 50 mM mM sodium acetate/D_2O at pD 4.75 for fPME) to obtain a concentration of 1% (w/v). Samples were stirred until the pectin was completely dissolved. Each buffer contained 1 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid, sodium salt (DSS; Merck, Darmstadt, Germany). Buffering of the solutions maintained optimum pH conditions for the pPME and fPME enzymes.

Immediately before the experiments were conducted, a small quantity of the stock enzyme solution was added to 1 mL of the appropriate cooled pectin-containing buffer to obtain a final enzyme concentration of 1.5×10^{-3}% (w/v) for pPME and 5.5×10^{-5}% (w/v) for fPME. After mixing, 300 μL aliquots of the resulting solution were transferred to the rheo-NMR cell and the control cell. The samples were briefly degassed by placing the cells under vacuum and sonicating them for a few seconds. This was done to prevent the formation of air bubbles during the experiments, which would be detrimental to the quality of the NMR spectra. This step was also considered important to eliminate air/water interfaces as potential sources of protein conformational change.

NMR spectroscopy

All NMR spectroscopy of samples placed under shear was performed using a Bruker Avance 500 MHz NMR spectrometer. A Bruker Avance 700 MHz NMR spectrometer was used to collect data for the unsheared control samples. To obtain ^{1}H 1D NMR spectra at 3 min intervals, one scan was acquired every 90 s, and consecutive scan pairs were concatenated into one spectrum. Each spectrum used a spectral width of 10.3 kHz and 64 k points. Presaturation using a field strength of 20 Hz was used for water suppression of the residual HOD signal. D_2O was chosen as the solvent to minimize the residual signal from the aqueous protons that persists after solvent suppression. Such a signal renders integration of neighboring peaks inaccurate. All experiments were conducted at 303.2 K based on a standard temperature calibration using the ^{1}H NMR spectrum of ethylene glycol. All spectra were processed with Bruker's Topspin software, and spectra from a particular experiment were phased and integrated identically. The deesterification process was monitored by measuring the increase in area of the methanol methyl proton signal relative to that of the methyl signal of the DSS reference in each of a series of NMR spectra recorded over the duration of the experiment. The clearer resolution of this signal compared to the signals from the pectin methyl groups made it preferable for analysis. DSS was used as an internal reference to avoid any possibility of the methanol/DSS peak heights varying between the rheo-NMR cell and the control cell assembly due to small variations in the volume of each cell. Control tests showed that DSS had a negligible impact on the enzyme kinetics under the conditions of the experiments.

Shearing protocols

Shear and control experiments were performed simultaneously because of the extreme sensitivity of the measurements to external variables. It was found in preliminary experiments that when different aliquots that originated from a single enzyme stock solution and had been frozen for storage, or a single aliquot that had spent different times at room temperature after thawing, were used, small (5–10%) but easily detectable differences in the enzymatic reaction rate were observed. In this setup, one portion of a sample served as a control in a 700 MHz spectrometer, and another portion was placed in the NMR Couette shear cell (but with no shear applied) in a 500 MHz instrument. Thin-walled, 5 mm NMR tubes with 3 mm NMR tube inserts were used for both portions.

We were unable to perform in situ experiments with the NMR spectrometer at average shear rates $>$ ~300 s^{-1} because the stepper motor tended to stall when we attempted to rotate the long drive shaft at high frequency. Therefore, to investigate faster rates, we developed a benchtop Couette shear cell consisting of a shorter cell shaft that enabled concentric rotation at higher shear rates corresponding to up to 1570 s^{-1}. For consistency, the thin-walled, 3 mm and 5 mm NMR tube setup was used again.

A disadvantage of the benchtop shear cell was that we could not use NMR to monitor the reaction in real time; however, we were able to obtain a spectrum during an experiment by transferring the sample to a conventional NMR tube. Although a number of studies have highlighted the reversibility of shear-induced conformational changes upon cessation of shear (21,25), we expect that in this case, any significant changes that occurred during shearing of the sample in the benchtop cell would have left their mark by differences in the degree to which the polysaccharide chains would have been demethylesterified.

In addition, this cell required the use of a waterbath, which necessitated accurate matching to the temperature used in the NMR experiments. To that end, a control sample was placed in the spectrometer at 303 K and spectra were recorded, and another sample was placed in the benchtop shear cell with the waterbath set to various temperatures around a nominal 303 K under no shear. The samples (pectin plus pPME) were left for 300 min in their respective cells, after which the benchtop shear cell sample was transferred to the NMR spectrometer for analysis to ascertain whether the demethylesterification reaction had progressed to the same extent as that in the control sample, i.e., whether both samples had undergone the same enzyme kinetics and therefore had been held at identical temperatures. The 300 min delay between the addition of pPME and the recording of spectra ensured that both samples were safely within the linear portion of the product versus the time region of the reaction.

RESULTS AND DISCUSSION

Initial NMR spectra

Pectin deesterification was readily observed in NMR spectra via the narrow peak at ~3.3 ppm, corresponding to the

methyl protons of the liberated methanol. The increase over time of this peak's intensity was mirrored by the decreasing intensity of the peak from the methyl protons of the esterified sugar moieties (Fig. 2). The large intensity and narrow shape of the methanol peak made it ideal for accurate integration. Indeed, the quantification of this peak in NMR experiments carried out on pectin samples in which all of the methylester groups had been released via alkaline saponification at high temperature has been used as a method of determining the DM of the pretreated polysaccharide (47).

Quiescent mesurements of enzyme activity

Data from the no-shear control experiment using a sample of pectin and pPME showed the rate of methanol liberation over time (Fig. 3 *a*). Fig. 3 *b* shows the corresponding change in DM of the substrate. Initially, the methanol concentration increases linearly with time, and then it slows down and apparently levels off. This was expected, as PME generally follows a Michaelis-Menten model (48).

Michaelis-Menten model

Michaelis-Menten kinetics is a simple model that describes enzymatic action. Although the time course is not usually fitted directly, the reaction rate, V, of an enzyme as a function of the substrate concentration, $[S]$, is easily written as (48):

$$V = V_{\max}\frac{[S]}{[S] + K_m} \tag{3}$$

This equation provides important information about the maximum reaction rate, $V_{\max}$, and the Michaelis constant, K_m, which reveals the substrate concentration at which the reaction rate is half its maximum value. Therefore, to investigate this relationship (Eq. 3) further, we carried out several additional experiments as a function of pectin concentration

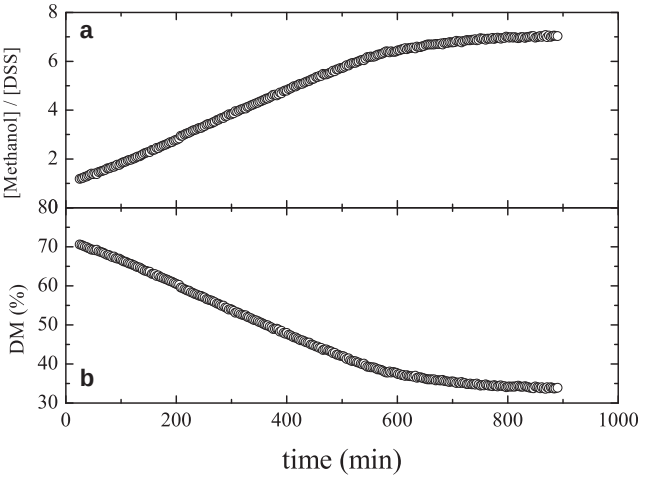

FIGURE 3 Control experiment showing (*a*) the rate of methanol liberated over time after the addition of pPME to 1% pectin as described in the text, and (*b*) the corresponding DM decrease.

(1.5%, 2%, 3%, and 5%). For each experiment, the reaction rate was obtained from the gradient of the linear region of the product evolution. Although the 95% confidence limits in the rates obtained from the linear regression analyses of the data ($R > 0.99$) were all <1%, error bars are illustrated on the figure that reflect the range obtained from triplicate experiments. Subsequently a Lineweaver-Burke plot of $1/V$ vs. $1/[S]$ was generated (Fig. 4), yielding $1/V_{\max}$ and $K_m/V_{\max}$ from a simple fit to Eq. 4, and allowing K_m and $V_{\max}$ to be determined as (100 ± 30) mM and (1.5 ± 0.2) × 10^{-3} mMs^{-1}, respectively (95% confidence intervals obtained from the fitting procedure), consistent with the results of previous experiments (33):

$$\frac{1}{V} = \frac{1}{V_{\max}} + \frac{K_m}{V_{\max}}\cdot\frac{1}{[S]} \tag{4}$$

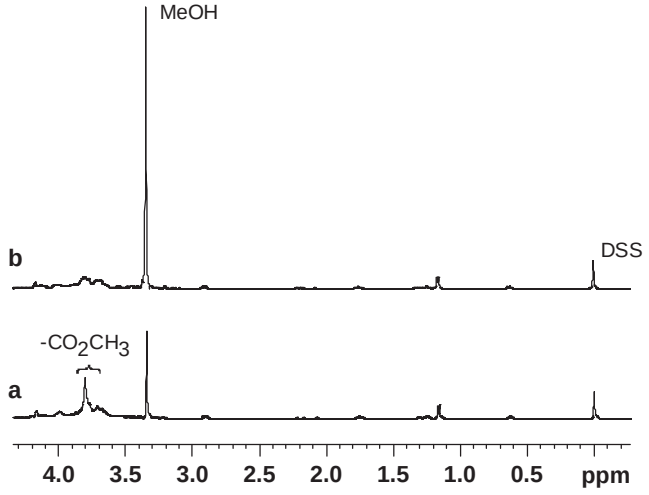

FIGURE 2 NMR spectrum showing peaks from the methylester group $-CO_2CH_3$, methanol MeOH, and DSS, (*a*) 30 min after the addition of pPME (the first spectrum of the series) and (*b*) after 14.5 h.

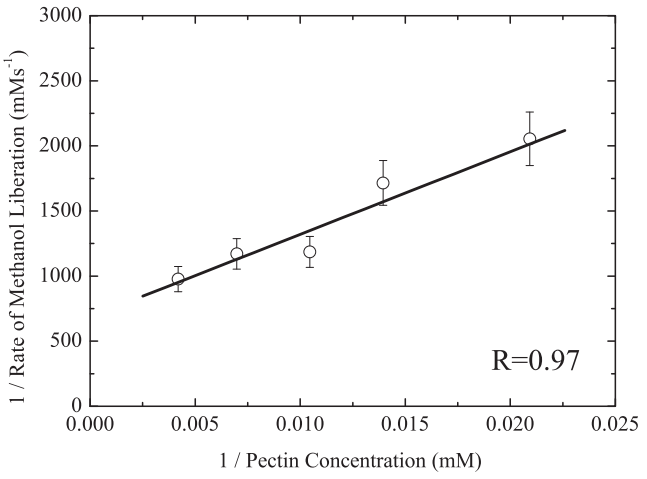

FIGURE 4 $1/V$ vs. $1/[S]$ plot for experimental data obtained for pPME action on a pectin substrate of varying concentrations. The solid line is a fit as described in the main text.

Simultaneous control experiments for shearing experiments

Before the shear experiments were conducted, a control experiment (Fig. 5 *a*) was carried out to demonstrate that experimentally indistinguishable enzyme kinetics could be obtained when recorded simultaneously on two separate spectrometers, as described in the Materials and Methods. As can be seen in Fig. 5, indistinguishable conditions could be generated in both spectrometers, providing confidence that any differences arising between the kinetics measured in the two spectrometers after the shear cell was turned on would indeed be due to the effect of shear.

Measurements of enzyme activity: comparison of quiescent and sheared samples

When shearing experiments were carried out (Fig. 5 *b*), an average shear rate of 240 s^{-1} was applied to the sample inside the NMR couette shear cell, and the remainder of the experiment was run under otherwise identical conditions to control experiments. It is apparent that no significant change in the enzyme activity rate was observed. Linear regression analyses carried out on the first 400 min of both data sets ($R > 0.99$) yielded rates that were indistinguishable within the returned 95% confidence intervals, and furthermore it was ascertained that rate changes of >5% would clearly be distinguishable, giving deviations at 400 min several times the standard deviation of the data. This seems to imply that shear did not significantly change the conformation of the enzyme or the pectin chains, or interfere with the interaction of pPME with pectin. Therefore, we conclude that there was no measurable change in the Michaelis-Menten parameters under shear compared with the control experiments. It is also clear that the sheared sample data were somewhat scattered in comparison with the control data. This was attributed to small mechanical vibrations from the stepper motor that were transferred to the sample cell via the ~1 m long drive shaft.

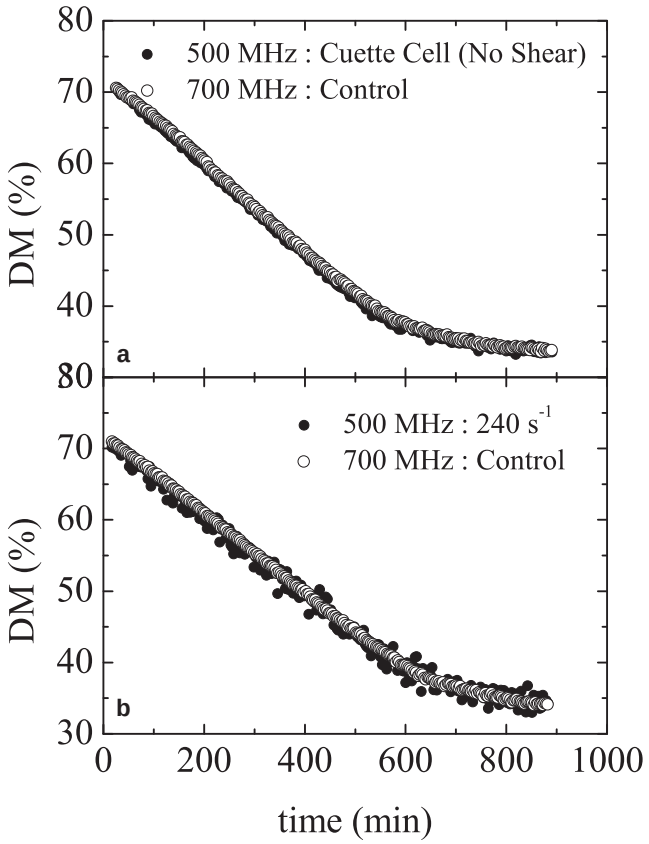

FIGURE 5 (*a*) Control experiment showing the action of pPME on 1% pectin, under an optimized protocol as described in the text, with a control sample, and a sample in the NMR Couette shear cell but with no shear applied. (*b*) Shearing experiment showing the action of pPME on pectin when sheared at 240 s^{-1}, and compared with the results of a simultaneously measured quiescent control sample.

Shearing experiments using a benchtop Couette shear cell

We were unable to perform in situ experiments in the NMR spectrometer at average shear rates > ~300 s^{-1} because the stepper motor tended to stall when we attempted to rotate the long drive shaft at higher frequencies. Therefore, to investigate faster rates, we developed a benchtop Couette shear cell consisting of a shorter cell shaft that enabled concentric rotation at higher shear rates corresponding to up to 1570 s^{-1}, as described in the Materials and Methods. Once consistent conditions between the benchtop and control cells were achieved, we carried out a further experiment using a shear rate of 1570 s^{-1} with otherwise identical conditions to those conducted in the NMR shear cell. However, despite the larger shear rate, the results (Fig. 6 *a*) showed no

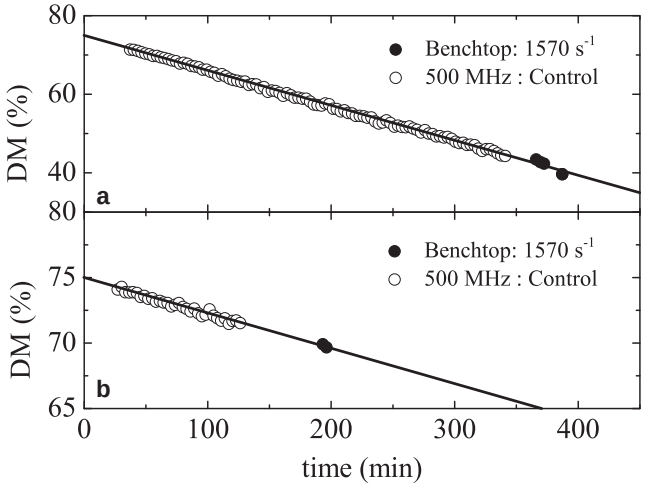

FIGURE 6 (*a*) Shearing experiment showing the action of pPME on 1% pectin when sheared at 1570 s^{-1}, and compared with the results of a simultaneously measured quiescent control sample. (*b*) Shearing experiment showing the action of pPME on a 5% pectin sample (five times the usual concentration) with an average shear rate of 1540 s^{-1}, compared with the results of a simultaneously measured quiescent control sample.

significant change in the enzyme activity rate, again implying that shear flow did not create substantial conformational changes in the macromolecules, or interfere with the enzyme-substrate interactions over this period.

Shearing experiments at higher concentrations

Additional shearing experiments were carried out using a 5% (w/v) pectin concentration with the benchtop Couette shear cell (Fig. 6 *b*). This was motivated by a previous study of DNA conformational dynamics as a function of the Weissenberg number, W_i, which is the dimensionless ratio of the applied shear flow rate to the polymer's natural relaxation (4). It was found in that work that at high viscosities, the amount of polymer chain extension increased as shear and W_i increased, due to an increase in the velocity gradient and therefore the net hydrodynamic forces (4). As described in the Introduction, for the experiments we carried out in water, the Weissenberg number for both the protein and the polysaccharide components is orders of magnitude < 1. In the concentrated system, the polysaccharide chain dynamics would be expected to be slowed by perhaps something approaching a factor of 1000, as estimated from the large change in viscosity of about that order, giving a Weissenberg number approaching one. However, even under these conditions, shear had no significant effect on the measured enzyme activity (Fig. 6 *b*).

Effect of shear on the enzyme activity of fPME

Further experiments were performed using fPME in place of pPME. We hypothesized that the random-acting nature of fPME could imply a less stable enzyme-substrate complex (as compared to the processive pPME), and that therefore its action on pectin might be more susceptible to the effects of shear. However, shearing experiments on pectin and fPME samples run at an average shear rate of 240 s^{-1} (Fig. 7) showed no detectable changes in enzymatic rate, further reinforcing the idea that shear does not create conformational changes in the macromolecules involved or interfere with the enzyme interactions under these conditions.

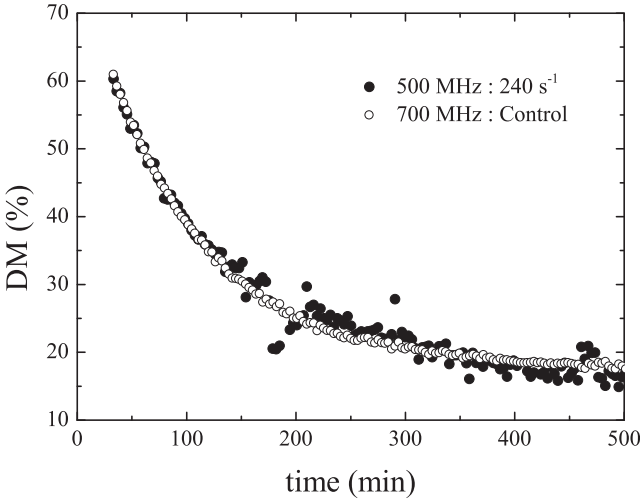

FIGURE 7 Shearing experiment showing the action of fPME on 1% pectin with an average shear rate of 240 s^{-1} applied as described in the main text, and compared with the results of a simultaneously measured quiescent control sample.

CONCLUSIONS

In this work, we used ^{1}H NMR to follow the kinetics of the liberation of methanol from the methylesterified polysaccharide pectin by PME enzymes. By using this methodology and varying the substrate concentration, we were able to show that the action of these enzymes is reasonably described by the Michaeles-Menton model. To make simultaneous measurements of the enzyme action using neighboring spectrometers, we mixed a single enzyme preparation (thawed from a single aliquot) with substrate and then immediately split the sample into two portions. This formed the basis of control and shear experiments at 240 s^{-1}, and the results obtained from each instrument were demonstrated to be indistinguishable within experimental uncertainties. Precise comparison was made possible by identical sample preparation, accurate temperature calibration, and degassing of samples.

Two different pectin demethylesterifying enzymes (one of plant origin and one of fungal origin) were used. Although the two enzymes exhibited different action patterns, in all cases the shear applied to the sample did not produce a measurable effect on the enzyme action. In addition, experiments carried out using a benchtop shear cell at a shear rate of 1570 s^{-1} also showed no detectable effect on the rate of methanol liberation. This implies that, at least for these predominantly β-sheet proteins and this polysaccharide, the shear conditions applied in this work did not significantly influence the intermolecular interactions or induce any significant relevant conformational changes in the macromolecules.

The null results of this study are in line with current theoretical predictions, but in contrast to some experimental results obtained with other proteins. Clearly, not all proteins will behave identically, and therefore it would be presumptuous to draw general conclusions from this one study. It remains to be seen whether the apparent discrepancies in the results in the literature can be resolved within a consistent framework, but promising avenues to be pursued will no doubt include careful comparisons of cell designs (flow characteristics and surface materials), the sensitivities of different detection modalities, and the different shear sensitivities of specific proteins.

It should also be noted that although two proteins of similar function were chosen here, this was by design. Although the catalytic activity performed in the active site is indeed equivalent for both proteins, and their sizes are extremely similar, it is well known that these proteins interact differently with their polysaccharide substrate.

Therefore, we reasoned that by comparing these proteins, we could isolate this interaction (i.e., the strength of binding) as an important factor if any difference was seen. However, these two systems provide a worthy starting point because of their distinct mechanisms of action, and the contribution that their characterization under shear makes to the ongoing debate over the influence of shear on medium-sized globular proteins. A broader understanding may result when all studies are considered together. The rheo-NMR technique described here may prove useful in the study of shear by allowing molecular detail to be resolved in structures that are reversibly deformed under shear flows. The search for such systems forms part of our ongoing work.

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